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DOES THE GONADOTROPIC HORMONE INDUCE ANTIBODIES OR ANTIHORMONES?

By FELIX SULMAN,* M.D.

CORRECTIONS

Vol. 63, No. 6, June 1, 1936

Page 816, 4th paragraph, first sentence, the words, "*and the acetyl polysaccharide of Pneumococcus I which likewise reacts with A immune sera (13)*," should be omitted.

Vol. 64, No. 6, December 1, 1936

Page 889, 16th line from bottom, for 52.1 read 56.4; for 22.7 read 27.0. 13th line from bottom, for 43 read 52. 7th line from bottom, for 13.0 read 17.8. 6th line from bottom, for 29 read 40.

Page 890, 3rd line from the top, for 12.5 read 13.3. 4th line from the top, for "This value, approximately 34 per cent of the total circulating serum protein, agrees very well with those recorded for dogs 3 and 4," read "This value is approximately 36 per cent of the total circulating serum protein. During the initial depletion week, 38.0 gm. of serum protein were removed from this animal." 20th line from top, for 30 to 40 read 35 to 55.

Page 895, 2nd line in 3rd paragraph under Summary, for 30 to 40 read 35 to 55.

and Schacter's report on the immunological properties of gonadotropin. A study of antithyrotropic activity containing ample references was recently given by Rowlands and Parkes (1936). Another recent paper was published by Werner (1936).

Engel, Smith, McPhail, Twombly and Ferguson, Fluhmann, H. Ehrlich, Braudt and Goldhammer) it has been established that in protracted application of prolan the effect upon the ovaries soon diminishes, until finally--within 2 months--there is no effect upon the ovaries at all. This phenomenon has been explained by Collip as being due to the effect of antihormones. The serological qualities of such prolan-resistant animals or those immunized in another way have been examined by us.

The question of the antigenic function of the anterior pituitary has been studied by Witebsky and Behrens, and by Ehrlich. Witebsky and Behrens, when immunizing with lipid extracts of the posterior lobe of the pituitary gland, obtained specific antibodies, which also reacted with lipid extracts of the brain. But by administration of lipid extracts derived from the anterior pituitary no organ specific antisera could be obtained. Experiments of this order tell us nothing about the antigenic function of prolan, the lipid extracts containing no prolan at all.

Ehrlich experimented with praepitan (Sanabo-Chinoin), a preparation derived from the urine of pregnant women, containing the gonadotropic hormone (prolan). He comes to the following conclusions: "We succeeded in gaining specific antibodies against the gonadotropic hormone of the anterior pituitary. In the same way we achieved a strict separation between the two endocrines of the same portion of the pituitary gland, the gonadotropic and the thyrotropic one, by purely serological means."

Quite independently of Ehrlich in 1934 we began to study the antigenic function of prolan. In the beginning our results were similar to those of Ehrlich. But when we used more purified prolan preparations we obtained,--to state the outcome in advance--results quite opposite to his. After examining and extending the results of Ehrlich in the course of the years 1935 and 1936 during a long series of experiments with 52 rabbits, we are today in a position to state: Prolan has neither the quality of an antigen nor that of a hapten,² and the results which Ehrlich looked upon as specific reactions of prolan are only reactions of the non-specific urine antigen, to be found in many

² Results similar to ours were obtained in the meantime by Bachman, Eichbaum and Kindermann, Brandt and Goldhammer.

urines and already described by Mertens, Schattenfroh, Zuelzer, Landsteiner and von Eisler, Friedenthal, Michaelis and Fleischmann, Přibram, Doerr and Pick, Uhlenhuth and Schulz, Friedberger and Suto, Sulman, Ehrlich. According to Savaré the excretion of colloids like this is increased during pregnancy.

EXPERIMENTAL

The Antigens.—The following preparations were used for immunizing *in vivo*:

TABLE I

Antigens in Vivo

1. Purified prolan (I.G.)
2. Crude prolan (I.G.)
3. Prolan (commercial preparation, I.G.)
4. Prolan with swine serum as carrier (*Schleppersubstanz*, Sachs) (coupled through combination by 1 hour's contact in the incubator at 37°C.)
5. Praepitan (Sanabo-Chinoin)
6. Antex (Løvens, Copenhagen)
7. Preglandol (Hoffmann-La Roche)
8. Menopause-prosylan (gonadotropic hormone derived from the urine of women at the menopause).

For the examination *in vitro* we also used other preparations, listed in Table II. As the different antigens contain a plurality of antibody formers corresponding to the way of derivation, and their competition may be decisive in the animals experimented upon as also *in vitro*, we show in Table II which components of antigen are contained in the different preparations.

The Antisera.—The antisera were derived from rabbits into which were injected intravenously, at intervals of 4 to 6 days, 100 to 500 r. u. (rat units) of the antigen in question (Table I) in 1 to 5 cc. of distilled water or physiological salt solution. Before injection, control tests were taken of the blood, which always reacted negatively with the antigen. 1 week after the 4th injection the 1st examination for antihodies was performed; this was repeated, especially when the result of the complement fixation was negative, until after the 16th injection. In case 8 of the animals did not show antihodies even after the 16th injection, the preparation was assumed not to have the quality of an antigen. In addition we considered it important that both male and female animals were immunized. We also treated male and female castrates with prolan, since after the removal of the effector organ the effect upon the blood could be expected to be an even more intense one. But we could not determine any difference between the various specimens of experimental animals.

The antigen-antibody reaction was performed in the fashion of complement

TABLE II
Antigens in Urine

Antigen	Manufacturer	Source material	Antigen components
Purified prol-an	I.G.	Pregnancy urine	Prolan A, B
Crude prol-an	I.G.	" "	Prolan A, B, urine anti- gen
Prolan (commercial preparation)	I.G.	" "	Prolan A, B, urine anti- gen (smallest quan- tity)
Proprilan	Sarstedt-Clintell	" "	Prolan A, B, urine anti- gen
Prosynyl	Organon	" "	" "
Menopausal prosynlan	Produced by our selves	Menopausal urine	Prolan A, B, synprolan*
Anter	Laroche	Mare Mare	" " " "
Luteinizing	Hi-Lite	" "	" " " "
Luteal serum	Sarstedt	" "	Prolan A, B, synprolan, serum protein antigen
Glandurolan	Hi-Lite	Pituitary glands of animals	Prolan A, B, synprolan
Preglan-101	Hoffmann-La Roche	" "	Prolan A, B, synprolan and other hormones of the anterior pitui- tary
<i>Extracts according to Zondek's method of concentrating prolan from urine et</i>			
(a) persons suffering from carci- noma	Produced by our selves	Human urine	Prolan A (varying), urine antigen
(b) healthy persons	" "	" "	Prosynlan (in infinitesi- mal quantities), urine antigen (in changing quantities)
(c) pregnant women	" "	" "	Prolan A, B, urine anti- gen (in varying quan- tities)

* The nomenclature introduced into literature by Zondek is used here:

Follicle-stimulating hormone: prol-an A.

Luteinizing hormone : prol-an B.

Synergic factor : synprolan.

The combination of these three hormones is called: prosylan.

† Method according to Zondek: A measured quantity of filtrated morning urine is shaken with 4 times the volume of 96 per cent alcohol; then it is allowed to stand for at least 12 hours. After this it is centrifugalized, the precipitation is washed with ether, allowed to dry and water to any desired quantity is added. The portion soluble in water can be used.

fixation. The methods were those usual for the Wnssermann reaction, while using half the doses.³

Furthermore we investigated⁴ whether we could make a special sensibility towards prolan appear by cutireaction (formation of a halo, necrosis following preliminary intracutaneous, subcutaneous and intravenous treatment). The results were uncertain. We could obtain the cutireaction as well with prolan as with extracts of urines derived from non-pregnant women and from men, and therefore practically free from prolan.

The serum of pregnant women (2nd and 3rd months of pregnancy), and that of those 1 day and 1 month postpartum were likewise examined for prolan antibodies by the method of complement fixation. The result was negative. Fluhmann, Brandt and Goldhammer, who examined such sera for antihormones, also had negative results.

1. Immunization with Purified Prolan.—15 rabbits were injected with 100 to 1000 r.u. of pure prolan intravenously. After 16 such injections the antisera did not react either with the antigen or with other urine extracts containing prolan.

Result.—Pure prolan is no antigen.

2. Immunization with Crude Prolan.—8 rabbits were injected with 100 to 1000 r.u. of crude prolan intravenously. After 4 to 6 such injections the antisera reacted with the antigen, with the boiled antigen, with urine extracts of individuals suffering from carcinoma and with several normal urine extracts. The reaction was negative with some other normal urine extracts, with human serum, pregnancy serum and with prolan (trade packages), in the concentration quoted in Table III, as well as with prosylan preparations of bovine and of equine origin.

Result.—Crude prolan produces antibodies not specifically effective against prolan, for they also react with the boiled antigen solution which is certainly free from prolan (Zondek and others). The positive reaction with urine extracts derived from healthy persons shows that prolan, if not purified, produces antibodies against the so called human urine antigen. Therefore the antiserum of crude prolan does not react with pure prolan, nor does it, obviously, with human serum protein.

³ We are indebted to Dr. J. Gurevitch who rendered the performance of the complement fixation reactions possible in the Hadassah Laboratory.

⁴ At the instance of Dr. M. Michael and in cooperation with him.

3. *Immunization with Prolan (Commercial Preparation).*—In our laboratory were 13 rabbits which for months received prolan solutions (commercial preparation 100 to 1000 r.u.) daily or twice a week subcutaneously or intravenously.

After about 2 months an effect upon the ovaries could no longer be observed, as Zondek demonstrated by numerous laparotomies.⁴ According to Collip and to

TABLE III

Complement Fixation of a Rabbit Antiserum (4 Dose) after 5 Immunizations with 2500 r.u. of Crude Prolan in All

Antigen solution, quantities	1 cc	15 cc	75 cc	5 cc	25 cc	1 cc
Crude prolan native	++++	++++	++++	+++	++	—
Crude prolan boiled	++++	++++	++++	+++	+	—
Pure prolan	—	—	—	—	—	—
Prolan commercial preparation ⁵	—	—	—	—	—	—
Mer. povidone prolylan	—	—	—	—	—	—
Preglar 1:1	—	—	—	—	—	—
Antex	—	—	—	—	—	—
Unconcentrated antiserum, 1000 r.u. antigen, 1 cc	0.1 cc.	0.15 cc.	0.2 cc.	0.15 cc.	0.1 cc.	0.05 cc.
Carbin in a ventrolull	++++	++++	++++	++++	+++	—
Normal cases, ♂ and ♀	++++	++++	++++	+++	++	—
“ “ “ “ “	—	—	—	—	—	—
Yeast in ventrolull	0.1 cc.	0.1 cc.	0.075 cc.	0.05 cc.	0.025 cc.	0.01 cc.
Human serum (normal)	—	—	—	—	—	—
Pregnancy serum	—	—	—	—	—	—
Control of antigens in double the doses	—	—	—	—	—	—

* Concentrated solutions (500 r.u.) yielded positive results too, but the use of highly concentrated antigens involves non-specific reactions.

Fluhmann this fact can only be explained by the effect of antihormones, for they state that the blood of such animals when simultaneously administered with prolan, inhibits the production of the anterior pituitary reactions I, II and III (follicle stimulation, blood points and luteinization).

In other words, animals continuously treated with prolan acquire the faculty of decomposing the prolan by protective ferments and of rendering it ineffective.

⁴ Zondek (1935), and experiments not published hitherto.

Our sera produced through prolonged treatment with prolan did not react in the complement fixation either with prolan or with any other antigen (normal urine extract, crude prolan, etc.), but 1 cc. of them paralysed the gonadotropic effect of 10 r. u. prolan in immature rats. As a result, the view which Goldherger and Ehrlich expressed in 1935, that the antihormone reaction is an antigen-antibody reaction, can be refuted.

Result.—Sera abounding in antihormones against prolan do not contain antibodies against prolan. The antihormones must be ranged in the ranks of the dissociating protective ferments and have nothing in common with the complement-fixing antibodies.

TABLE IV

*Complement Fixation of a Rabbit Antiserum ($\frac{1}{2}$ Dose) after 8 Immunizations with 4000 r.u. Pure Prolan in All
(In Coupling through Combination with a 10 Per Cent Swine Serum Dilution)*

Antigen in decreasing quantities	20 u.	10 u.	7.5 u.	5 u.	2.5 u.	1 u.
Prolan	—	—	—	—	—	—
Prolan-swine serum native	++++	++++	++++	++++	+++	—
Prolan-swine serum boiled	++++	++++	++++	++++	+++	—
Swine serum native in a corresponding dilution	++++	++++	++++	++++	+++	—
Swine serum boiled in a corresponding dilution	++++	++++	++++	++++	+++	—
Control of antigens in double the doses	—	—	—	—	—	—

4. *Immunization with Prolan and with Swine Serum.*—We demonstrated in the preceding experiments that prolan is no antigen, but there remained the question whether it is a haptene. The haptene is fully effective *in vitro*, but *in vivo* it is restricted in its immunizing potency, for it is only able to develop antigenic qualities in a more or less tight coupling with a molecule-enlarging carrier substance (*Schleppersubstanz*, Sachs). In this way it can be masked in such a fashion that, although native to the body, it can act as if foreign to the body and can incite the production of antihodies. The carrier was always at the disposal of the prolan in the shape of the urine antigen, but it is known through the papers of the school of Sachs that, in the plurality of antigens produced by coupling, a concurrence of the components in the body of the animal studied will occur. The competition of the antigens may lead to the domination of one of the components, whether the ways of reaction in some animals have a tendency in favour of the one component,

or the carrier substance is dominant *a priori*. Since swine serum is known to be especially suitable as an indifferent carrier, the coupling through combination was used: 500 r.u. of prolan in 5 cc. of 10 per cent swine serum dilution, 1 hour in the incubator at 37°C., according to the example of Landsteiner, Klopstock and Selzer. The results are to be seen in Table IV.

The antiserum does not react with prolan, but it does so with swine serum and with the combination swine serum + prolan, also when boiled, without any difference in the scope of reaction. This experiment was performed with the serum of 4 rabbits and always had a similar course with only one exception, in which the rabbit turned out to be altogether refractory. The inhibition by excess (*Ueber-schussverminderung*, Landsteiner-Hallban) in the reaction of swine serum with addition of prolan failed.

Result.—The coupling of prolan with swine serum as a carrier substance makes it evident that prolan is not able in this form either, to produce antibodies reacting only with prolan. Accordingly prolan is neither an antigen nor a haptene.

3. Immunization with Praeipitin (Santal-Chinoin).—These experiments were performed in support of the above mentioned experiments of Ehrlich and their results took a conformable course. But as to the interpretation of the results we arrive at conclusions the exact opposite of his. 4 rabbits were treated with injections of 100 to 500 r.u. of praepitin. After the 4th injection the animals, which had received doses of 200 to 300 r.u. (i.e. after an application of 800 or 2000 r.u. respectively (Table V)), showed a positive complement fixation with all prolan preparations of human origin examined; with pure prolan alone they did not, neither with prolan (commercial preparation, I. G.) in the concentration quoted in Table V, nor with menopause-prosylan. Furthermore there was no reaction at all with prosylan preparations of bovine and of equine origin. Urine extracts produced according to the prolan precipitation method of Zondek, from normal human beings and from pregnant women,⁴ also did not react, normal human serum and pregnancy serum likewise. But one urine specimen of a pregnant woman and all the 10 urine extracts from patients with progressive carcinoma and a positive albumen finding in the urine did react (urine antigen reaction) (Table V).

The appearance of urine antigens is not bound exclusively to moribund conditions or to urines positive to sulfosalicylic acid, but varies according to unknown laws, even daily in the same individual, as we can state as an incidental finding in this paper, and as has been already demonstrated by other authors (see above). Boiling of the antigens could not change the given results (Table V). Thus our findings do not differ in any way from those of Ehrlich. But if Ehrlich had examined prolan preparations of other than human or urinary origin, or if he had

⁴ See Table II.

TABLE V

Complement Fixation of a Rabbit Antiserum ($\frac{1}{2}$ Dose) after 4 Immunizations with 2000 r.u. Praepitan in All

Antigen in decreasing quantities	20 u.	10 u.	7.5 u.	5 u.	2.5 u.	1 u.
Praepitan native	++++	++++	++++	++++	+++	—
Pregnyl native	++++	++++	++++	++++	++	—
Crude prolant native	++++	++++	++++	++++	+	—
Praepitan boiled	++++	++++	++++	++++	+++	—
Pregnyl boiled	++++	++++	++++	++++	++	—
Crude prolant boiled	++++	++++	++++	++++	+	—
Pure prolant	—	—	—	—	—	—
Prolant (commercial preparation)*	—	—	—	—	—	—
Menopause-prosylan	—	—	—	—	—	—
Antex	—	—	—	—	—	—
Glanduantin	—	—	—	—	—	—
Lutocrescin	—	—	—	—	—	—
Luteoantin	—	—	—	—	—	—
Preglandol	—	—	—	—	—	—
Preparation of the anterior lobe of the pituitary gland	—	—	—	—	—	—
Urine extracts in decreasing quantities corresponding to native urine	0.5 cc.	0.25 cc.	0.2 cc.	0.15 cc.	0.1 cc.	0.05 cc.
4 normal cases, ♂ and ♀	—	—	—	—	—	—
2 " " " " "	++++	++++	+++	++	+	—
2 " " " boiled	++++	++++	+++	++	+	—
10 cases of progressive carcinoma	++++	++++	++++	+++	+++	—
10 cases of progressive carcinoma boiled	++++	++++	++++	+++	+++	—
2 cases of pregnancy	—	—	—	—	—	—
1 case of pregnancy	++++	++++	++++	++++	+++	—
Sera in decreasing quantities	0.2 cc.	0.1 cc.	0.075 cc.	0.05 cc.	0.025 cc.	0.01 cc.
Human serum normal	—	—	—	—	—	—
Pregnancy serum	—	—	—	—	—	—
Control of antigens	—	—	—	—	—	—

* Higher concentrations (500 r.u.) reacted positively.

proceeded with prolant preparations largely free from urine antigen, he would not have looked upon his reaction as one specific for the gonadotropic hormone. For the thyrotropic hormone (Schering) examined by him, which is not derived from

human urine but from the pituitary glands of animals, is no comparable control. On the contrary, only a really purified prolan or a prolan of non-human or non-urinary origin may be looked upon as such, and neither has been considered by Ehrlich in his paper. In our estimation, also, the fact that a praecipitan solution heated for 1 hour at 65°C. still had a positive reaction in Ehrlich's investigation should have indicated to him that the reaction in question was no prolan reaction, for at such a temperature prolan is already seriously damaged.⁷ Recently Brandt and Goldhauser, Eichbaum and Kindermann, and Bachman have also opposed the results of Ehrlich.

In a further experimental series we tried to inhibit the praecipitan reaction by an excess of prolan. This experiment failed and accordingly we could not demonstrate a semihaptenic function of prolan.

Result.—Prolan preparations which have not been freed from human urine antigen to the utmost degree are able to simulate a positive antigen function of prolan. Only investigation with purified prolan and with that of bovine and equine origin can serve as evidence that prolan does not possess either the function of an antigen or that of a hapten. On the other hand the positive results of the reaction with boiled prolan prove that the simulated antibody reaction cannot be due to prolan, the latter having been destroyed by boiling.

6. Immunization with Antex (a Protylan Preparation of the Lévens Komiske Werke).—As it was now quite certain that pure prolan (A + B) cannot exert antigenic functions, the question remained whether perhaps the combination with synprolan (synergic factor) is able to cause an increase in the size of the molecule which would be sufficient to produce its constitution as an antigen or hapten.

For this purpose, in the beginning, we investigated menopauze-prosylan and preglandol, both of which preparations had proved to be exceedingly effective in subcutaneous titration in rats and rabbits. Unfortunately, our experiments had no result, the rabbits studied dying after the 2nd injection in consequence of the relatively high toxicity of the preparations when applied intravenously. But immunization with antex (a highly effective prosylan preparation, derived from the blood of mares) turned out to be successful and informative. 8 rabbits were treated with 100 to 500 m.u. of antex intravenously up to 16 times. None of the animals produced any antibodies. This fact is all the more interesting, for it proves that the combination of prolan (A + B) with synprolan cannot be looked

⁷ According to von Euler and Zondek, prolan becomes ineffective at 62°C. within 45 minutes up to 50 per cent; at a temperature of 67°C. within 45 minutes up to more than 90 per cent, this depending also upon the pH. Therefore we cannot state precisely how far the praecipitan of Ehrlich had been destroyed at a temperature of 65°C.

upon as an enlargement of the molecule, but probably only as a synergic effect, and it proves furthermore that no great difference in the size of the molecule can be ascribed to the synergic factor either, in comparison with prolán, and finally it shows that it is possible to purify an effective gonadotropic hormone preparation (derived from mare serum) from horse protein to such a high degree that the demonstration of the origin in a serological way is no longer possible.⁸

Result.—Prosyán has neither the effect of an antigen nor that of a haptene.

DISCUSSION

As we pointed out in the preceding pages, the phenomenon of the diminishing effect of ovary-stimulating factors upon protracted application can be explained through the production of antihormones. These antihormones must be looked upon as protective ferments in the sense of Abderhalden. Their effect is a dissociative but not a complement-fixing one, for we are able to paralyse *in vivo* and *in vitro* the gonadotropic effect of the ovary-stimulating substance through antihormone sera. The examinations of rabbits proved that no complement fixing antibodies, but only antihormones, can be produced by administration of gonadotropic hormones of various origins. On the basis of these examinations, we conclude that Collip's antihormones must be looked upon as protective ferments, but certainly not as antibodies. Furthermore we can state that the antihormones do not enter the urine (*cf.* also Brandt and Goldhammer). This suggests the supposition that the antihormones themselves are not hormones, since most of the sex hormones are to be found in the urine. The importance of the problem of antihormones for the protracted treatment of clinical cases will be further investigated.⁹ As to the specificity of the

⁸ Recently we have daily immunized a rabbit for 11 weeks with antex. The serum was free from antibodies (proved by the complement fixation) and free from antibormones (proved by the gonadotropic reaction in immature rats).

⁹ Recently we have examined the blood of a male patient who had undergone treatment with about 12,000 r.u. of prolán in the course of 9 months. 8 weeks after close of the treatment it proved to be free from antibormones and antibodies. These results can be explained through the lack of a blood reaction against a substance native to the body (*horrer autotoxicus*). The prolán used in this case was of pregnancy urine origin. Another female patient who had undergone treatment with 4200 r.u. of prolán in the course of 2 months failed to form antibodies and antibormones entirely.

antihormones, we have demonstrated that they work as specific for the species of origin (*cf.* Brandt and Goldhammer, Fluhmann, Bachman). According to unpublished investigations from this laboratory, the antihormone against pregnancy prolan does not exert an effect upon prosylan derived from the blood of pregnant mares (antex). Parkes and Rowlands only deny the specificity of origin. As to the organ specificity, opinions still differ; according to Collip and collaborators, and also Fluhmann, an antihormone against pregnancy urine prolan is not effective against human pituitary prosylan and *vice versa*. According to Brandt and Goldhammer, however, the antihormone against human urine prolan is also effective against human serum prolan, placental prolan and pituitary prosylan. This discrepancy has also still to be clarified.

SUMMARY

1. It is shown in 52 experiments upon rabbits that the purified gonadotropic hormone has no antigenic function--as an antigen or a haptene--either in the form of prolan derived from the urine of pregnant women or in the form of prosylan, derived from the blood of pregnant mares.

2. The experiments reported elsewhere which ascribe an antigenic structure to prolan, are due to confusion with the so called non-specific urine antigen (urine colloid).

3. Sera of rabbits which are abundant in prolan antihormone (Collip) also contain no antibodies against prolan. The antihormones against the gonadotropic substances must accordingly be considered as protective ferments (Abderhalden).

4. The employment of castrated animals has no influence upon the above results.

5. The lack of immunizingly effective components in pure prolan and prosylan (*a*) inhibits the possibility of serological diagnosis of pregnancy for the diagnostician; (*b*) protects the therapist against undesirable toxic and allergic secondary effects in intensive prolan treatment. The latter also is valid for pure prosylan preparations.

6. No prolan antibodies could be found in the blood of pregnant women in the 2nd and 4th month of pregnancy, or 1 day and 1 month postpartum.

7. Antihormone against prolactin of human origin has no paralyzing effect upon a prolactin preparation derived from the blood of pregnant mares (antex); there exists therefore a species specificity.

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AN ANALYSIS OF MITOSIS IN LIVER RESTORATION

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PLATE 1

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Sections of liver tissue taken during the process of hypertrophy following partial hepatectomy show varying numbers of karyokinetic figures, at times even as great as those seen in rapidly growing malignant tumors. This fact has been noted by all who have studied this phase of experimental pathology. The chief object of this investigation has been to correlate mitosis in this tissue with the known growth rate.

Higgins and Anderson (1) have shown that when the main lobes of the liver of the white rat are removed, the remnant, which comprises about 30 per cent of the original liver, begins at once to hypertrophy. The rate of increase in size is greatest during the 1st day of hypertrophy, and restoration is complete after about 3 weeks. Brues, Drury and Brues (2) reinvestigated this matter from the point of view of cell restoration and showed that there is no increase in hepatic cells during the 1st day of restoration, and that cell restoration, throughout, lags about a day behind tissue restoration.

A number of questions in regard to mitosis in liver restoration have never been satisfactorily settled. There is still considerable discussion as to whether the predominant form of cell division in this organ is karyokinetic or amitotic. Some writers have thought that mitosis was more frequent in the periphery of the lobules than elsewhere, or in cells recently developed from budding bile ducts, while others have failed to make such observations. Moreover some writers have assumed that in this and other tissues, the number of mitoses seen at any given time can be considered as an index of the growth rate. We have sought to establish answers to such questions as these.

Method

Inbred albino rats of the Slonaker strain were used in these experiments. Animals were chosen which weighed between 100 and 180 gm. Partial hepatectomies were done by the method described in another paper from this laboratory (2), at which time it was demonstrated that the residual fragment left in the rat was 31.6 ± 1.5 per cent of the total liver.

Animals were sacrificed at various time intervals, some under ether and some by decapitation after stunning. The liver was removed as rapidly as possible and thin slices were fixed in Zenker's fluid with 5 per cent acetic acid, in every case within 5 minutes of the animal's death, and in most cases within 1 or 2 minutes. This precaution was taken since Thüringer (3) has shown that mitoses become progressively less numerous in the human prepuce as the length of time elapsing between operation and fixation of tissue increases. No differences in the appearance or frequency of mitoses were observed between the livers removed under ether and those which were not subjected to the anesthetic.

After dehydration and clearing, the tissues were embedded in paraffin and sections 6 μ in thickness were cut, and stained with iron hematoxylin and eosin. A few sections, to be used for examination of mitotic spindles, were stained with phosphotungstic acid hematoxylin as described by Warren (4).

Sections in which chromosomes were to be examined were stained by the Feulgen reaction as described by Ludford (5) and counterstained with saturated picric acid.

In one series of animals in which it was desired to follow the incidence of mitosis over a period of time, biopsies were taken from the hypertrophying liver under brief ether anesthesia. The operative incision was reopened and a wedge-shaped snip of tissue between 1 and 2 mm. in size was cut from the edge of the organ. When successive bits were removed they were taken from widely separated places. Following biopsy in this fashion, there was no obvious reaction except locally in the affected area.

Mitoses were in every case counted under the oil immersion objective, and over an area containing at least 1000 hepatic cell nuclei. At least 400 nuclei were actually counted; and in sections of even thickness, other measured areas of the same size were assumed to contain (within the reasonable limits of error) the same number of cells. The incidence of mitosis is always expressed as per cent of hepatic cell nuclei dividing in a given area. Fig. 1 is given to show examples of the earliest and latest stages which were recorded as mitotic figures. Where no mitoses are recorded, it is to be understood that at least 2000 hepatic cells were counted. In many cases the phase of mitosis was noted, as prophase, metaphase, anaphase or telophase according to the judgment of a single observer. The greatest source of error here lies in the fact that some anaphases of which a small part was included in the section have doubtless been called metaphases. Since mitotic figures occupy, on the whole, a somewhat larger space than resting nuclei, it is likely that the absolute percentages of dividing cells found are actually a little high, but as all

counts were done on sections of the same thickness (6μ) this error should be the same in all cases.

RESULTS

An occasional mitosis (but never more than one in 10,000 to 20,000 cells) can be seen in resting liver. During the 1st day after partial hepatectomy few, if any, more can be seen. Near the end of this time, it can be seen that there is a very noteworthy variation in the size of nuclei, which are remarkably uniform in resting liver. This condition persists for the next 2 days, and then slowly becomes less striking. Although it is likely that the larger nuclei are most prone to divide, this cannot be observed directly.

No "abnormal mitoses" have been observed in our series. All hepatic cell mitoses in any given phase are about the same size. It has not been practicable to count the chromosomes in our material. Mitoses in the smaller bile duct cells are always much smaller than those in hepatic cells (see Fig. 2). No attempt has been made to count the mitoses in bile duct cells, but it seems that their incidence is roughly parallel to that of mitoses in hepatic cells, and likewise they are not seen during the 1st day.

On a small series of sections of actively dividing tissue stained by the phosphotungstic acid hematoxylin method, a few mitotic spindles in hepatic cells were chosen and drawn with the camera lucida, in order to estimate the angle of the spindle. In eight spindles conforming to the requirements laid down by Warren (4), that is, lying perpendicular to the line of vision, the angle was found to be between 60° and 70° , with an average value of 64° . This is a relatively narrow angled type of spindle. One of these spindles is shown in a photomicrograph (Fig. 3).

The question of the distribution of mitoses throughout the organ was investigated in a section removed 48 hours after operation. The mitotic incidence in this section was over 3 per cent. About one-half of a complete transverse section of a lobe was mapped out with the aid of a camera lucida and all the mitoses were drawn in place, and the phase of each was noted. The drawing was then divided into 300 equal squares, 379 mitoses were found, and the number of squares containing respectively no mitoses, one mitosis, and so on, was counted.

This number of squares (out of the 300) containing each finite number of mitoses is shown in Table I, together with the theoretical number of squares which should have the same number within. This calculation is done assuming an entirely random distribution, according to the

TABLE I

No. of mitoses	Actual No. of squares	Theoretical No. of squares
0	79	84.8
1	118	107.0
2	60	67.8
3	33	28.6
4	8	9.0
5	2	2.3
6	0	0.5
	300	300.0

TABLE II
Mitosis Counts

Specimen No.	1	2	3	4	5	6
Time after operation, days.....	3	3	3	2	1	1
	4	1	4	20	41	26
	7	4	1	11	41	28
	8	2	4	15	45	23
	5	3	7	10	40	29
	8	2	6	12	41	40
	7	4	5	16	39	24
	4	2	4	12		
	4	2	3	13		
	9	4	4			
	6	4	3			
Mean.....	6.2	2.8	4.1	13.6	41.2	30.0

Poisson distribution. The results show that there is a very close approximation to the theoretical distribution in sampled areas of the size chosen; and hence that in this specimen there is no systematic tendency of mitoses to occur in groups.

There is in addition no greater tendency of mitoses to appear near

bile ducts than elsewhere. It was also thought worth while, since it seemed on casual observation that cells in the same phase of mitosis (especially prophases) tended to be somewhat grouped, to investigate this from the same map. Hence the phase of mitosis of the nearest dividing cell to each of the 33 prophases was noted. Four prophases, 22 metaphases, five anaphases, and two telophases were found. But three prophases would have been expected on a random basis.

TABLE III
Percentage of Mitosis in Hepatic Cells

Approximate time after operation			
1 day	2 days	2 days	3 days
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
2.0	2.2	0.2	0.28
2.4	1.36	0.2	0.36
0.0	1.75	4.4	1.1
1.2	1.2	0.85	0.9
2.0	0.8	0.11	0.62
1.5	2.0	0.5	0.8
0.9	0.35	1.0	0.5
1.3	0.9	1.2	1.1
4.12	2.4	0.8	0.7
3.4	1.3	0.9	0.6
4.2	0.0	1.2	0.41
1.7	0.0	0.9	0.75
3.0	0.05	0.0	0.1
	1.1	1.6	
	1.15	0.0	
	0.4	0.0	
	1.8	0.7	
Mean....2.13	0.97		0.63

A further series of counts from scattered parts of six specimens of regenerating liver are shown in detail in Table II, as a further indication that the distribution of mitoses is reasonably uniform throughout the organ. Each mitosis count is on 1000 adjacent liver cells.

Table III, which shows the percentage of mitoses in 60 consecutive livers taken at periods between 24 and 75 hours after operation, indicates that there is a great variation in the frequency of mitosis in a series of regenerating livers which should be growing at approximately the same rate.

These livers were removed within 3 hours of the 1, 2, and 3 day intervals following operation, and the nearest integral number of days in each case is shown. The mean value for each period is shown, and we note that the individual figures range in each case from zero to two or more times the mean value. The significance of these values will be discussed later.

In view of this extreme variability in mitosis counts done at random

TABLE IV
Percentage of Hepatic Cells in Mitosis

Time	Actual No.									
	1	2	3	4	5	6	7	8	9	10
Day	percent	percent	percent	percent	percent	percent	percent	percent	percent	percent
19					0					
20						0				
21					0	0				
22		0								
23			0							
24		0	0	8.15(1)	0.10	0.13				
25	1.23(2)			3.40						
26	3.67(3)									
27				0.53					2.14(4)	
28				0.69						1.29
29									2.03(5)	
30										1.35
32										0.56
38									0.54	2.08(6)
46								1.36		
48							0.93		0.91	
49										2.72(7)
50							0.74	0.72		
54							1.09	0.88		

Figures in parentheses refer to specimens in Table V.

on regenerating livers, it seemed desirable to establish if possible that the mitosis count in a single liver, from which biopsy specimens were taken from time to time, varied similarly.

Table IV shows that this is definitely the case, and also that mitosis can be considered as beginning rapidly after the 24th hour post-operatively.

Since in some of the experiments the mitotic count rises with time,

while in others it falls, there is evidently no constant effect of the operative procedure which could be considered as an important source of error. It is reasonably certain also, from Tables I and II, that the variations are not due to unequal distribution of mitoses throughout the entire liver.

In Table V is shown the distribution of mitoses with respect to phase, in seven of these specimens. It appears that the percentage of mitoses in each phase shows slight changes from time to time, probably dependent on the fluctuations from hour to hour in the total mitoses. There is nothing in these specimens or in any others which we have examined to suggest that a large number of mitoses begin simultaneously throughout the liver.

TABLE V
Percentage of Total Mitosis in Each Phase

Specimen No.....	1	2	3	4	5	6	7
Time after operation, hrs.....	24	25	26	27	29	38	49
Prophases, per cent.....	20.5	15.0	18.7	8.9	15.0	11.6	4.1
Metaphases, per cent.....	48.2	51.2	48.3	55.6	52.5	65.3	51.0
Anaphases, per cent.....	22.2	18.8	20.9	24.4	22.5	15.4	30.6
Telophases, per cent.....	9.1	15.0	12.1	11.1	10.0	7.7	14.3
Total mitoses counted.....	176	80	91	45	40	26	49

DISCUSSION

Having the raw data above, it is now interesting to consider what is the correlation between mitosis rate and the growth rate of the liver cells as a whole. Since, according to previous work (2), it has been shown that during the interval from 24 to 72 hours after operation the number of hepatic cells in the liver increases from 33.5 to 65.5 per cent of the number of cells originally present, we may assume that for every 1000 cells present at the beginning of this period, 955 mitoses occur during the next 2 days. Since, however, each mitosis count represents the number of mitoses present at a certain time proportional to the number of cells present at that moment (not to the number present at the beginning of any finite period), it is clear that we must have recourse to the calculus in order to correlate these counts with the

growth rate. We must first find a simple empirical expression for the cell growth curve.

In the first part of Table VI is shown the number of cells present in the liver at various postoperative intervals, as per cent of the cells originally present, from data in another paper (2). We are evidently dealing with a rate which is maximal during the 1st day of cell restoration (24 to 48 hours after operation) and decreases constantly thereafter. Moreover, experimental work of various kinds suggests that restoration is correlated with the portal blood passing through the remaining liver parenchyma after operation (6-8).

Whether or not this explains cell multiplication as well as tissue hypertrophy, we may consider as a first approximation for purposes of a mathematical expression, that the rate of cell increase begins maximally and then is constantly retarded so that it remains proportional to the number of cells yet to be formed, approaching the original

TABLE VI
Number of Cells in Liver

Time post operative, days	1	2	3	4	6
Actual No., per cent	31.5	52.0	65.5	72.0	81.0
Calculated No., per cent	33.4	52.1	65.6	74.8	87.8

cell number as a limit. This type of growth is commonly seen by the embryologist, and would have the general formula:

$$N = C(1 - e^{-kt}) \quad (1)$$

where N represents the number of added cells, t is time, and C and k are constants. According to this formula, when $t = 0$, $N = 0$, and when $t = \infty$, $N = C$, while between these values the increase in N is proportional to an exponentially depreciating quantity representing the number of cells not yet formed. Since N at the beginning is finite, t must be finite also, and we must neglect that part of the curve before the beginning of cell restoration. Since C is the limiting value of N , we may put it equal to the number of cells originally present and express N and C in such terms that $C = 1$. If now we take the first few figures in Table VI, we find that they are satisfied by formula (1), expressing t in days after operation. This can be done by putting

$k = 0.33$ and adding 0.23 to t , in order to set the initial rate at the observed value. This gives the theoretical values in the table.

Thus the empirical formula fits the data through the 3rd day. Beyond this point the growth appears to be retarded more than would be expected from our simple assumption. This might be due to the development of retarding substances or to the fact that the rate may not be directly proportional to the excess blood flow, or to other factors. No reasonable revision of the formula, however, would make any important difference in the growth curve through the 3rd day.

Olivo and Slavich (9) have studied the mitotic incidence in the chick embryo heart, and on correlating this with the growth rate show that the number of mitoses seen is the number which should occur during a 38 minute period; they conclude that this is the average duration of cell division. We have made further calculations for the purpose of

TABLE VII

Interval, days.....	1	2	3
M/A , per cent per day.....	63.9	30.5	17.1
Mean percentage mitosis.....	2.13	0.97	0.63
A , days.....	0.0333	0.0318	0.0369
Duration of mitosis, min.....	48.0	45.9	53.1

establishing a similar period in our material. Differentiating formula (1), and inserting constants, we obtain:

$$\frac{dN}{dt} = 0.33e^{-0.33(t+0.23)} \quad (2)$$

Now, if we let M represent the proportion of cells in mitosis at any one time and A represent the average duration of a mitosis, we may consider that M/A represents the instantaneous rate of increase in the cell number divided by the number of cells in the liver at that moment. Therefore,

$$\frac{M}{A} = \frac{dN/dt}{N}, \text{ or } M = \frac{A \times 0.33e^{-0.33(t+0.23)}}{1 - e^{-0.33(t+0.23)}} \quad (3)$$

According to this, the values of M in terms of A have been estimated for the 1, 2, and 3 day intervals after operation, and are shown in

Table VII, together with the values found in Table III, and the resulting calculated mitosis time.

The most probable mean duration of our mitoses would, therefore, be 49 minutes. This time is a little longer than that which Olivo and Slavich established for the chick embryo heart. Among the sources of error which must be considered are these: (*a*) the personal equation in the matter of judging the earliest prophases and the latest telophases; (*b*) the fact that many mitoses cover more space than resting nuclei and so are likely to be seen in a thin section with greater proportional frequency; (*c*) the fact that the 1 day counts in our material were mostly done at the beginning of mitosis, when in certain cases there seems to be a very high initial count which falls off rapidly (see No. 4 in Table IV). The last two of these possible errors would make the true counts lower than the estimates and so increase the mitosis time. It is worth noting that Olivo and Slavich made their estimates on a basis of 2 day intervals, and that the several figures for these intervals range from 33 to 68 minutes. Moreover, any increase in average cell size before the 20th day would, if allowed for, raise the mitosis time above their estimates.

Various observers have timed the visible duration of mitosis in tissue cultures and have obtained figures of the same order of magnitude as our own. Lewis and Lewis (10) obtained figures of about 30 minutes to 2 hours for fibroblasts, and in the Walker rat sarcoma 338 they found the duration to be about an hour (11). Strangeways (12), with embryo fibroblasts, found the duration to be 34 minutes.

It would be vain to attempt to state the intermitotic period in our cells, since we have no way of knowing whether cell increase is by several divisions of a few cells, or by a few divisions of all cells.

The extreme variability in mitosis rate at various times seems well shown in our data. It appears from figures given by Olivo and Slavich (13) that the same is true in less degree in tissue cultures. As far as can be seen in Table IV, there is little uniformity with respect to time in the various animals of our series, except for the consistent absence of mitosis during the 1st day. There is no reason to suspect that there is an instantaneous mitogenetic stimulus at any time, since the mitotic figures seem in all cases well distributed throughout the several phases; nor is there any evidence of local mitogenetic effects or of local varia-

tions due to differences in the blood supply of the central and peripheral parts of the lobule. Moreover, the random scattering of mitoses does not support the idea that the new tissue is to any great extent derived from bile duct cells.

It is very interesting that no mitosis occurs for about a day after operation. It can be noted that mitosis does not take place until the cells have, on the average, increased to about one and one-half times their usual volume; but it is likewise true that in fasted animals the cell increase continues at nearly the usual rate while the size of the liver is actually decreasing (2). It can be seen that the hepatic cells fill with lipoids at about the time when division begins. The only noticeable morphological change in the cells before the onset of mitosis is that the nuclei become increasingly variable in size and chromatin content. This visible premitotic stage, however, occurs relatively late in the 1st day. The latent period in our material is analogous to the period before growth in fresh explants, which has been found by various observers to increase with the age of the explanted tissue (14). Liver explants, however, will not proliferate at all if taken from animals beyond the latter half of embryo life (15). It has generally been thought that the latent period in explants is the time required for the cells to become dedifferentiated, but the hypertrophying liver in our experiments shows none of the characteristics ordinarily attributed to undifferentiated tissue.

The mitosis rate near the beginning of activity in the liver (21.3 per 1000 cells) is strikingly similar to that in early embryo growth (22.5 in the 2 day chick heart (9)) and to that in well growing cultures of mesenchyme (between 2 and 3 per cent (16)). One might think that this represents the maximal rate at which certain tissues can undergo prolonged cell growth, although we have seen that the instantaneous rate in individual cases can be much higher.

SUMMARY

1. Following partial hepatectomy in the rat, there is a latent period of 1 day during which the rapidly growing organ shows no increase in cell number. Mitosis then begins rapidly, following a brief premitotic period of visible nuclear changes.

2. It can be shown that the increase in cell number during the ensuing 48 hours follows a formula of the type $dN/dt = ke^{-kt}$; beyond this time it is retarded more than this simplified formula would predict. The average mitosis rate at 1, 2, and 3 day intervals after operation follows the same formula; from this the duration of each mitosis is calculated to be about 49 minutes. It is not necessary to assume that amitotic division plays an important part, and no such divisions have been seen by the writers.

3. The percentage of cells in mitosis in a single hypertrophying liver varies widely from hour to hour, so that a single mitosis count tells nothing about the growth rate. The fluctuations occur at different times in different livers. It appears that no great number of mitoses begin or end simultaneously.

4. Mitoses are evenly distributed throughout the liver and throughout each lobule; there is no preponderance near the bile duct cells.

5. The mean initial mitosis rate (at 24 hours after operation) is 2.13 per cent, and it diminishes from then on. This rate is very similar to that in early embryo heart and tissue cultures of mesenchyme. In individual specimens the rate can be over 8 per cent. This rapid rate occurs without signs of cell dedifferentiation.

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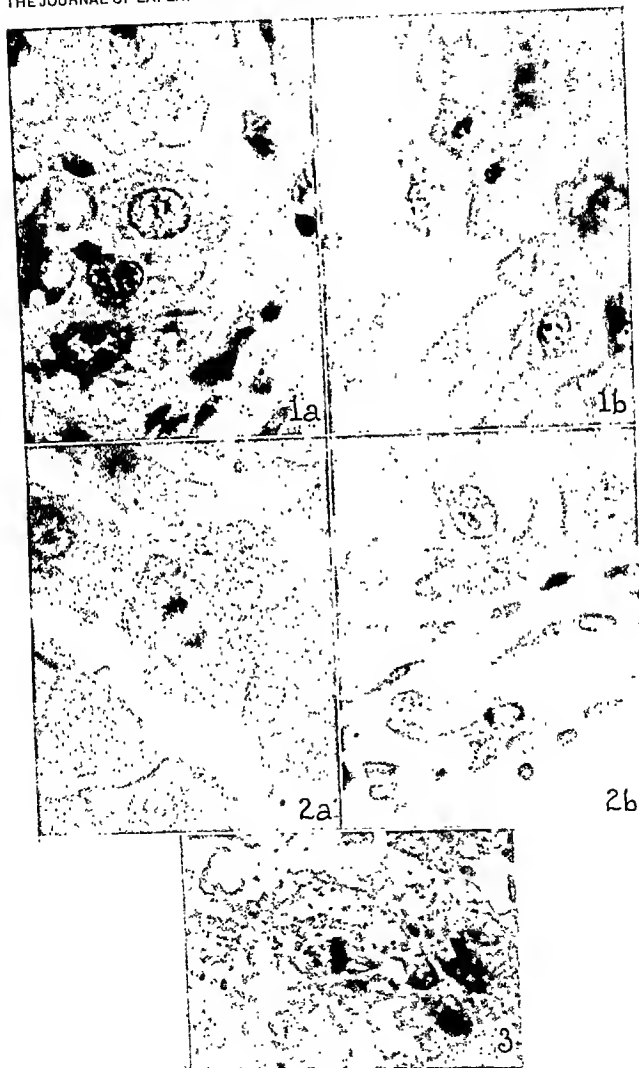
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EXPLANATION OF PLATE 1

FIG. 1. Early prophase (*a*) and late telophase (*b*). These figures represent the approximate limits between which cells have been counted as in mitosis. Iron hematoxylin-eosin. $\times 900$.

FIG. 2. Anaphases in hepatic cell (*a*) and in bile duct cell (*b*). Iron hematoxylin-eosin. $\times 900$.

FIG. 3. Spindle in hepatic cell mitosis. Phosphotungstic acid hematoxylin. $\times 900$



(Brues and Marble: Mitosis in liver restoration)

THE CAPILLARY SUPPLY IN NORMAL AND HYPERTROPHIED HEARTS OF RABBITS*

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PLATES 2 AND 3

(Received for publication, August 3, 1936)

It is common experience to find at the autopsy table hearts which have failed during life, but which show no myocardial abnormality except hypertrophy. It is generally believed that hypertrophy enables the heart to perform its work more advantageously in the presence of certain alterations in physiological conditions. This belief, however, is not supported by sufficient factual evidence and there is no conclusive proof that an hypertrophied heart is actually a better performer than a heart of normal size. Indeed, the question may be raised whether hypertrophy might ultimately become a handicap to the heart and actually contribute to failure.

If, during the process of hypertrophy, the mass of muscle substance should increase without a corresponding increase in the total number of capillaries, the concentration of capillaries per unit of tissue would be decreased. If changes in blood flow could not compensate for this, the heart might suffer from an impairment in the exchange of metabolic substances. It has been shown by Goldenberg (1), Tangl (2), and others (3, 4) that hypertrophy is associated with an increase in the diameter of the individual muscle fibers. Since the capillaries of the heart, for the most part, accompany the fibers in a parallel course, it is obvious that such an increase in the cross sectional diameter of the fibers would push the capillaries farther apart, and, as a result, increase the distance between the wall of a given capillary and the periphery of its region of supply.

* Read at the meeting of the American Society for Clinical Investigation in Atlantic City, May 4, 1936.

The present study was undertaken in order to determine whether hypertrophy of the rabbit heart is accompanied by such a decrease in the concentration of capillaries or whether the concentration is maintained at a normal level by the birth of new capillaries at sufficient rate to keep pace with the increase in muscle mass. An answer was sought from an analysis of the following material, which was obtained from a series of hypertrophied hearts controlled by a similar series of normal hearts: (a) measurements of the diameter of the muscle fibers; (b) counts of the number of capillaries per square millimeter in the transverse plane of the fibers; and (c) determination of the ratio of the number of capillaries in the tissue to the number of fibers. If it is assumed that in hypertrophy there is no change in the number of fibers, a multiplication of capillaries would be indicated by an increase in this ratio.

Methods

Choice of Animals.—The rabbits of both the experimental and control groups were selected from a variety of breeds, and not more than three were taken from any one litter. There was a fairly even distribution of the sexes in each group.

Operative Procedures.—Cardiac enlargement has been produced experimentally by the creation of various artificial defects in the circulatory system. The most notable are: (a) aortic insufficiency (5); (b) arteriovenous fistula (6); (c) removal of buffer nerves (7, 8); and (d) stenosis of the pulmonary artery (9, 10). Each of these was tried in the present study and each usually produced hypertrophy, but the first was employed in the majority of instances because it necessitated no complicated operative procedure and a satisfactory hypertrophy resulted. Intravenous sodium pentobarbital anesthesia was used, and sterile technique was employed in all operations.

An insufficiency of the aortic valve was produced by the rupture of one of its leaflets. This was accomplished by the introduction of a sound with a bulbous tip through the left carotid artery. (The puncturing of two leaflets almost invariably resulted in the death of the animal within 4 to 48 hours.) The rabbits were killed after 2 to 5½ months had elapsed. In several instances it was noted that false valves had formed in the aortic wall just above the ring of the valve. Arteriovenous fistulae were produced by the creation of a lateral anastomosis between the left carotid artery and jugular vein. The method used for removal of buffers was essentially the same as that of Kremer, Wright, and Scarff (8), except that in most instances the operation was done on both sides at once.

The procedure which was followed in order to produce stenosis of the pulmonary artery in these experiments represents a modification of previously described methods. Reid (9), and Holman and Beck (10) applied occluding bands to the

pulmonary artery in dogs. In their experiments a certain amount of right ventricular hypertrophy occurred, but a permanent stenotic orifice could not be made small enough to insure a markedly increased resistance to the flow of blood.

In the present study a small aluminum band was placed around the pulmonary artery of rabbits when they were about 6 weeks old and weighed from 300 to 500 gm. The band, when used on these very young rabbits, caused a constriction which, although not small in comparison to the size of the vessel at that time, was a relatively narrow stenosis when the rabbits had become full grown. The band, which was of sheet aluminum 0.3 mm. thick, was at first made with an inside diameter of 2 mm., but it was found that the rabbits died after reaching a weight of about 800 gm. With bands which had a diameter of 3 mm. the rabbits grew to maturity without apparent impairment in health. The operative approach was by an incision through the midline of the sternum in the region between the first and third ribs. Artificial respiration was provided by a small tube inserted through an incision in the trachea. Closure of the sternal and tracheal incisions was made with silk. The rabbits were killed 7 to 10 months later.

Injection and Fixation of Hearts.—The method which was used to inject the capillary bed was essentially the same as that devised by Wearn (11). The heart was perfused with oxygenated Locke-Rosenheim solution at a pressure of 50 to 60 mm. of Hg for adult rabbits and 20 to 30 mm. for very young ones. After the heart had begun to beat, a 2 per cent suspension of Berlin blue was injected through the rubber tubing above the aortic cannula by means of a needle and syringe. When the heart appeared uniformly blue, glacial acetic acid was injected. This brought about immediate cessation of contractions and fixed the tissue.¹ Heart weight was determined after all excess fluid had been wiped from the heart and the cavities dried with cotton swabs. The heart was then placed in 10 per cent formalin for fixation. The cavities were first filled with the solution before the heart was immersed so that the natural contour of the ventricles would not become distorted.

Quantitations of capillaries and measurements of fibers could not be made without first controlling the shrinkage which generally occurs during the process of preparation of tissue for microscopic study. Measurements indicated that the tissue shrank about 2 per cent during the first few days in formalin and in a following 2 to 3 months an additional 1 per cent was added. It was considered that the shrinkage from fixation which did not exceed 3 per cent was small enough to be disregarded.

After the hearts had remained in formalin for 3 to 10 days, the auricles were removed and the capacities of the ventricles were determined with mercury. The two ventricles were next separated by the method of Herrmann (12) and each

¹ Registration of contractions on a smoked drum showed that the heart always stopped in complete relaxation. The ventricles, however, were not in a state comparable to physiological diastole when the cavities are distended with blood.

was weighed. Formalin fixation produced considerable weight loss and for this reason ventricular weights were corrected to their original weights before fixation.

Preparation of Sections.—At first attempts were made to prepare sections by the ordinary paraffin and celloidin methods, but it was discovered that the shrinkage during these processes was of such magnitude and subject to such variation that it could not be disregarded. A given section sometimes decreased to 60 per cent of its original area. Such reductions in area could have been corrected mathematically in each separate instance, had it not been apparent that in many sections, even when external dimensions changed very little, there was an internal shrinkage characterized by an increased separation of fibers from each other, or a massing of fibers into artificial bundles which left wide crevasses between. Measurements of external dimensions could not be used to correct for this type of shrinkage. The following method, which was ultimately adopted, produced no shrinkage: Four blocks of tissue, two from the midportion of the right ventricle opposite the septum, and two from the corresponding portion of the left ventricle, were removed from the formalin-hardened heart. These were cut in such manner that the large bundles of fibers which encircle the ventricles in the transverse plane were in cross section. They were washed in running water for 24 hours, placed in 10 per cent gelatin at 37°C. for 24 hours, followed by 20 per cent gelatin for a similar period, and then imbedded in the latter. After the gelatin had solidified, the blocks were hardened in 10 per cent formalin. Frozen sections, about 15 micra thick, were cut, stained in eosin and mounted from water without blotting. The edges of the gelatin were gently touched with absorbent paper, which drew the water from beneath the section and flattened it on the slide. Arabic sugar was used as a mounting medium. Capillary counts were then made immediately, since it was found that in such a preparation the Berlin blue tended to fade out of the capillaries.

Counts and Measurements.—All counts and measurements were done with an oil immersion objective and ruled micrometer ocular square, covering an area of the tissue which varied with the microscope from 3000 to 3500 square micra. In view of the fact that it was desired to determine the number of capillaries and fibers in pure muscle tissue, fields were selected for counting which did not contain connective tissue, arteries, or veins. Crevasses caused by any tears in the section were likewise avoided. Counts were made in areas in which the capillaries and fibers were cut in perfect cross section and in which the capillaries were completely injected, as shown by their even spacing and regular distribution. Except for the restrictions mentioned, no deliberate selection of fields was made. Another system of counting which was tried consisted of choosing adjoining fields in continuity, regardless of their content, in a straight line across the section. Deductions were made for the areas occupied by bare spaces, connective tissue, etc. This method gave results which compared favorably with those of the first method, but it was discarded as it was time-consuming and introduced an additional error in the estimation of blank spaces in a field. The chief criticism of the first method, which was adopted as routine, is that one may be prejudiced and un-

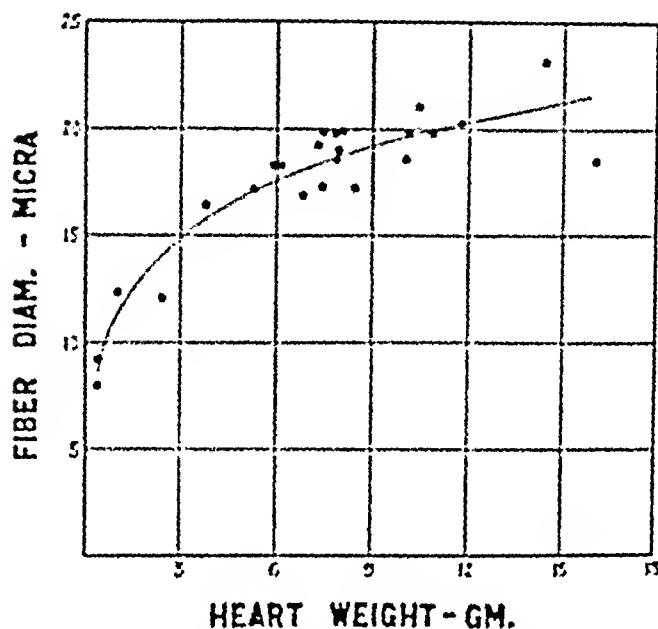
consciously choose fields with a high or low count as his expectation suggests. In all cases, however, the counts were made by at least two observers and often by three. There was almost always one observer who did not know the identity of the heart being counted and yet the counts of all observers agreed very well. The final average was calculated from a total of 120 to 240 fields counted in each ventricle. A fiber count was made simultaneously with the capillary count so that a ratio of the two could be obtained. It is probable that practically all of the capillaries were injected in those regions of the muscle which were selected for counting. In any event, if incomplete injection existed, it was undoubtedly present to an equal extent in both normal and hypertrophied hearts.

Measurements of the diameters of fibers also were made in areas where the fibers were in perfect cross section. Those fibers which were measured were selected in sequence as they touched a single straight line when the slide was moved laterally on the mechanical stage. Most fibers were ovoid in shape and therefore both long and short diameters were measured. From 160 to 240 fibers were measured in each ventricle. The long and short dimensions were first averaged separately and then an average of the two was computed to give the mean fiber diameter. In that the width of the myocardial fibers varies with systole and diastole, these measurements cannot be considered absolute. The number of fields counted to determine the average capillary and fiber count of a given heart, and the number of fibers measured to obtain a representative fiber diameter were found sufficiently large to reduce the probable error of the mean well below the limits dictated by accepted methods of statistics.

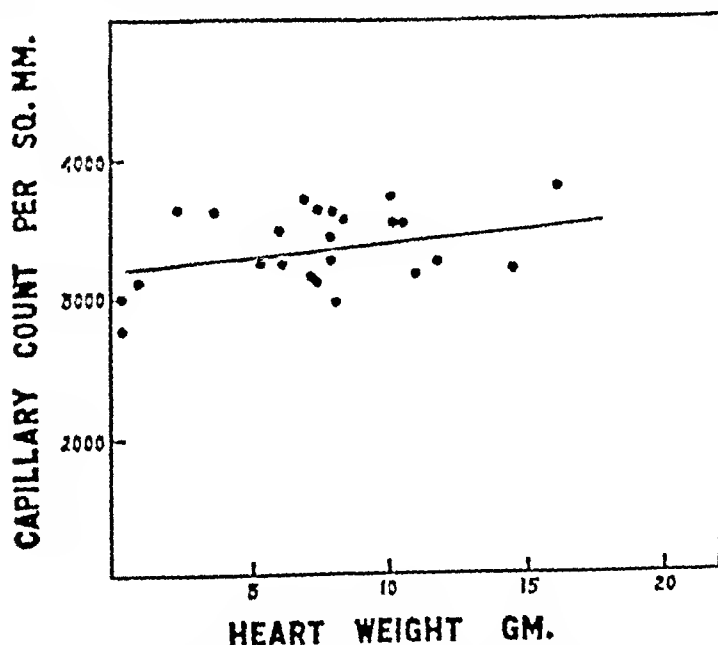
Normal Hearts

A number of hearts from both young and old rabbits at various ages were studied for the purpose of observing the changes in the capillary bed during the normal growth of the heart, and for the comparison of these changes with those occurring in the capillary bed during the process of hypertrophy.

Hearts were obtained from rabbits whose body weights varied from 55 gm. at birth to 5135 gm. in maturity. It was observed that the transverse diameter of the fibers became gradually larger with increase in size and weight of the heart (Text-fig. 1). This mode of growth was first demonstrated by Tangl (2) who found no evidence of multiplication of fibers after birth. The increase in the cross sectional area of an average fiber from birth to maturity was sevenfold and the increase in total fiber length was by estimation nearly sixfold. A sevenfold increase in cross sectional area would reduce the capillary supply to one-seventh of the original concentration if new capillaries were not formed during the growth of the heart. A formation of new capillaries did take place, however, for the capillary count per square millimeter remained essentially the same throughout the period of growth. Indeed, it was a little higher in some of the larger hearts (Text-

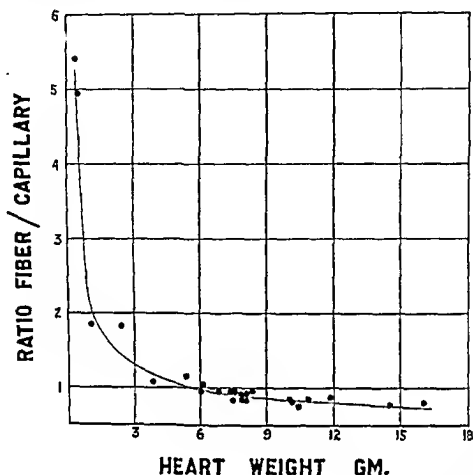


TEXT-FIG. 1. The influence of growth on fiber diameter (left ventricle).

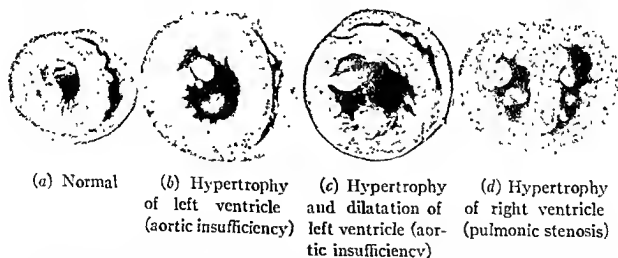


TEXT-FIG. 2. The influence of growth on the capillary count (left ventricle).

fig. 2). At birth there were about five small fibers to each capillary (Fig. 1), but as the fibers enlarged the capillaries multiplied until a ratio of one capillary to each muscle fiber was reached. In the larger adult hearts there were actually



TEXT-FIG. 3. The influence of growth on the number of fibers per capillary (left ventricle).



TEXT-FIG. 4. Cross sections of normal and hypertrophied hearts showing the effect of hypertrophy on the thickness of the walls of the ventricles and on the size of the chambers. Natural size.

a few more capillaries than fibers (Text fig. 3). The capillary count in the right ventricle averaged slightly lower than that of the left, the fibers of the former were slightly smaller than those of the latter, and the number of fibers in proportion to capillaries was somewhat higher. The lower counts in the right ventricle may have been associated with a tendency for the tissue of this chamber to be less compact than that of the left.²

Hypertrophied Hearts

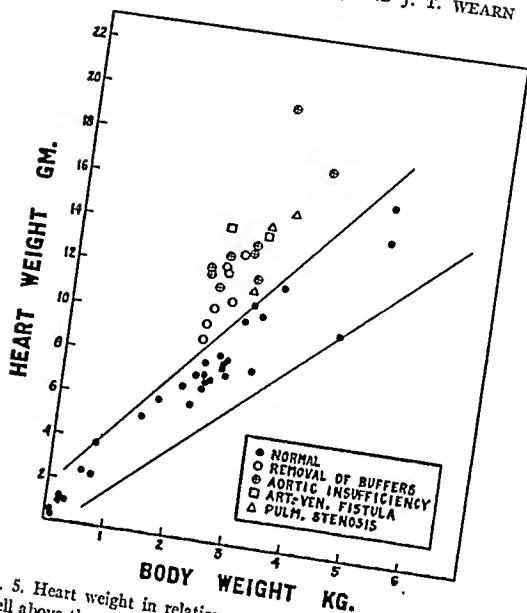
The criterion for hypertrophy was increase in heart weight as compared to body weight. The presence of the Berlin blue in the injected specimens, it was found, caused an increase in weight of 20 to 50 per cent. For this reason only injected hearts were used to establish a series of controls. It will be seen from Text fig. 5 that all of the hypertrophied hearts fell well above the normal range. The hearts of a few of the rabbits in which the vagus nerves were removed were not included in the study, for their weights were not definitely increased.

Observations on hearts in which hypertrophy was manifested primarily in the left ventricle are recorded in Table I (see also Text-figs. 6 and 7 and Fig. 3). It will be noted that the fibers were of larger diameter in the hypertrophied group than in the control, and the capillary count was reduced.³ The reduction in capillary count agreed fairly well with an expected figure which was calculated on the assumption that no new capillaries were formed, and that the pre-existing capillaries were pushed apart by fibers which increased in area from 361 to 493 square micra. (Fiber area was calculated by squaring fiber diameter.) This observation, along with the fact that the ratio of capillaries to fibers did not change, allows a fair conclusion that multiplication of capillaries did not occur.

The outcome was somewhat different when hypertrophy developed gradually in young growing rabbits as a result of pulmonic stenosis. Although this group was too small for proper statistical analysis, the

² The capillary counts of normal rabbit hearts in this study were somewhat lower than those reported by Wearn. This was undoubtedly due largely to the elimination of shrinkage in the present study and to the fact that we made no attempt to select areas in which the count was at a maximum.

³ The ratio of the long diameter of the fibers to the short diameter was almost identical with that of the normal hearts. In other words, the fibers increased in size equally in both dimensions.

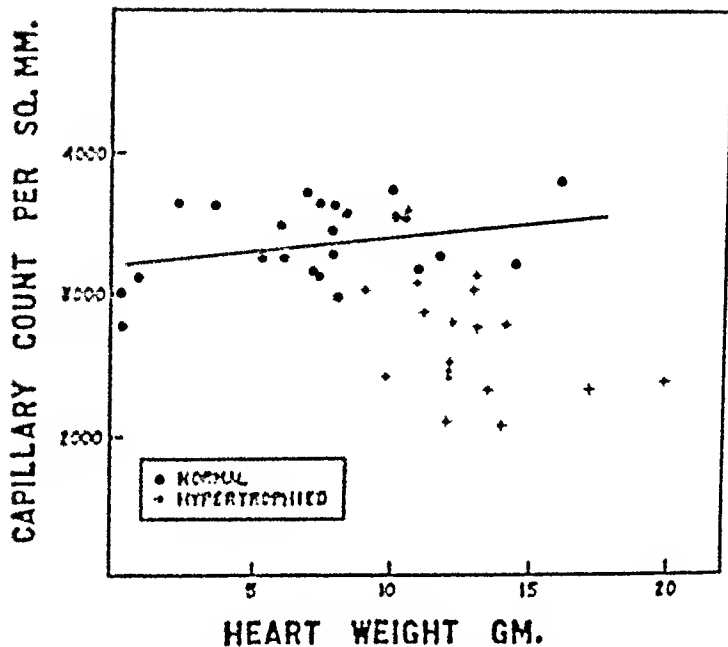


TEXT-FIG. 5. Heart weight in relation to body weight. The hypertrophied hearts fall well above the normal controls.

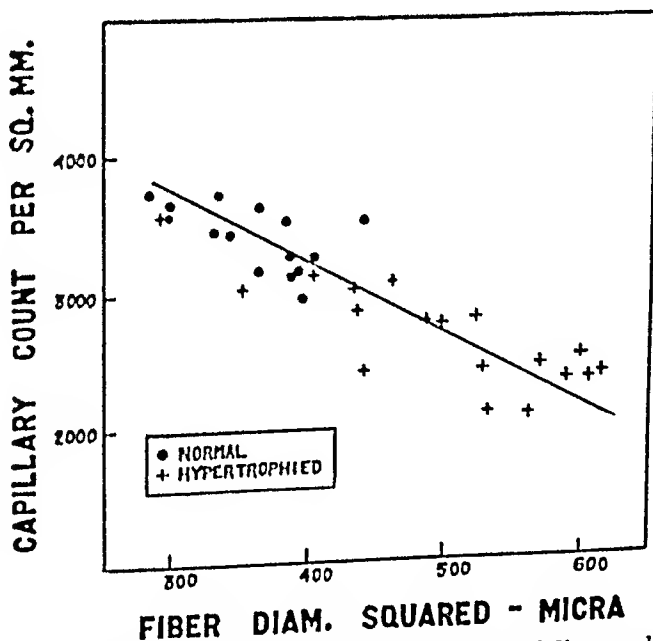
TABLE I
The Effect of Hypertrophy (Left Ventricle)

	Normal hearts (mean of 15 with probable error)	Hypertrophied hearts (mean of 18 with probable error)
Fiber diameter, <i>micra</i>	19.0 ± 0.20	22.2 ± 0.33
Capillaries per sq. mm.	3420 ± 39	2670 ± 62
Capillaries per fiber.	1.14 ± 0.013	1.16 ± 0.013

Limits of body weights of normal rabbits used for controls were 2050 to 4530 gm., average 2790 gm. Limits of body weights of rabbits with hypertrophy of the left ventricle were 2245 to 4055 gm., average 2710 gm.



TEXT-FIG. 6. The capillary count as influenced by hypertrophy (left ventricle). Note that the counts in the hypertrophied hearts fall below the range of the normal.



TEXT-FIG. 7. The relation between cross sectional area of fibers and capillary count (left ventricle). Coefficient of correlation = -0.89 ($P.E. \pm 0.025$).

findings were fairly uniform. The size of fibers in the right ventricle was well above normal, but the capillary count per unit area, although somewhat low, was not reduced to the expected figure and the ratio of capillaries to fibers averaged somewhat above the normal (Table II). The only tenable explanation for the failure of a reduction in the capillary count to the level which would be anticipated from the increased size of fibers is that there was a certain degree of multiplication of capillaries in response to the hypertrophy which in these hearts did not occur after the heart had reached a relatively mature state, but developed simultaneously with active growth.

Measurements of ventricular weight and fiber diameter indicated that in all of the experimental hearts except those with pulmonic

TABLE II

The Effect of Hypertrophy in Growing Rabbits with Pulmonic Stenosis (Right Ventricle)

	Normal hearts (mean of 15 with probable error)	Hypertrophied hearts (mean of 3)
Fiber diameter, <i>micro</i>	17.2 \pm 0.23	23.0
Capillaries per sq. mm.....	3310 \pm 59	2740 (Expected count—1850)
Capillaries per fiber.....	0.99 \pm 0.19	1.15

Limits of body weights of rabbits with pulmonic stenosis were 3025 to 3530 gm., average 3220 gm.

stenosis hypertrophy was not confined to the one ventricle, but was shared by the other to a smaller degree. The capillary count was reduced to an extent corresponding to the degree of hypertrophy.

It would be expected that the presence of any appreciable degree of dilatation would cause an elongation and narrowing of the muscle fibers. This might offset the effect of hypertrophy which produces thickened fibers. An analysis of the influence of dilatation as expressed by the ratio of chamber volume to chamber weight, however, disclosed the fact that increases in chamber capacity of the magnitude encountered generally affected the fiber size and capillary count to only a slight degree.

DISCUSSION

It is evident that the process of hypertrophy differed from that of growth in its effect on the capillary bed. It appears that some factor which was responsible for the genesis of capillaries during natural growth was not in evidence during hypertrophy. An indication of the presence of some stimulating factor which influences capillary formation during normal growth may be secured from the findings in the hearts with pulmonic stenosis. In these hearts, in which hypertrophy developed simultaneously with growth, the capillary count was higher than in those in which hypertrophy occurred after the normal growth process had nearly ceased.

In the mature group the period during which the hypertrophy developed was of sufficient length to allow the capillaries to multiply. It is hardly probable that if a longer period had been allowed before injection the results would have been any different. The capillary counts in general were no higher in hearts injected 5½ months after operation than in those after 2 months.

The obvious question to ask oneself at this point is whether or not the decreased capillary supply could embarrass the myocardium because of an impairment in metabolic exchange. An enlarged heart would be expected to consume and discharge greater quantities of metabolic substances than a normal one. An augmented blood flow might serve to supply the increased mass of muscle tissue, and might also compensate for the relatively sparse distribution of capillaries. It cannot be stated at present, however, whether an increase in blood flow really occurs in these hearts, or whether an increase, if present, can actually meet the demands of a larger amount of tissue with relatively few capillaries. The physiological significance of the present findings cannot be estimated without further data bearing on these problems.

Measurements of capillary diameter were not made. Simple inspection revealed no difference in size between the capillaries of the normal and those of the hypertrophied hearts. It should be kept in mind, however, that the postmortem appearance of a capillary is not necessarily an indication of its premortem state. It must also be remembered that even if capillary dilatation were demonstrated, this in itself would not be an indication of actual augmentation in the

volume flow of blood. The size of the other vascular channels, and the pressure within them must also be considered.

Findings in the hypertrophied rabbit heart cannot be applied to the human heart without some reservation. A study of the capillary bed in normal and hypertrophied human hearts is now in progress.

CONCLUSIONS

1. During normal growth of the rabbit heart, muscle fibers enlarge, and the capillaries multiply so that a relatively constant capillary supply per unit of tissue is maintained from the time of birth to maturity.

2. In cardiac hypertrophy the muscle fibers enlarge, but the capillaries do not multiply and, as a result, the capillary supply per unit of tissue is reduced.

3. The decreased concentration of capillaries in the hypertrophied heart would constitute an impediment to the adequate exchange of metabolic substances, but the seriousness of the impediment cannot be estimated without further physiological data.

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EXPLANATION OF PLATES

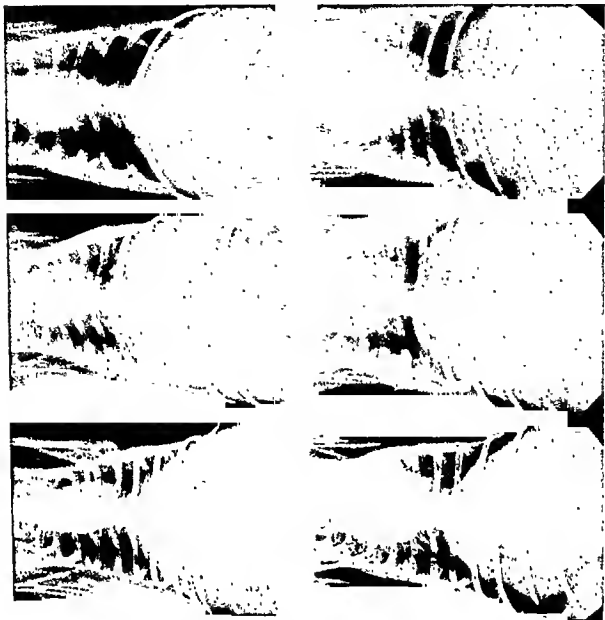
PLATE 2

FIG. 1. The capillary supply at birth (ventricular septum) $\times 500$. Note the small fibers and compare with Fig. 3a.

FIG. 2. X-ray photographs showing the increase in size of the cardiac silhouette after operative procedures. Top row, before operation—bottom row, after operation. (a) removal of buffers; (b) aortic insufficiency; (c) arteriovenous fistula.

PLATE 3

FIG. 3. The capillary supply of the normal heart (a) compared with that of an hypertrophied heart (b) (left ventricle). $\times 500$.



c

b

FIG. 2

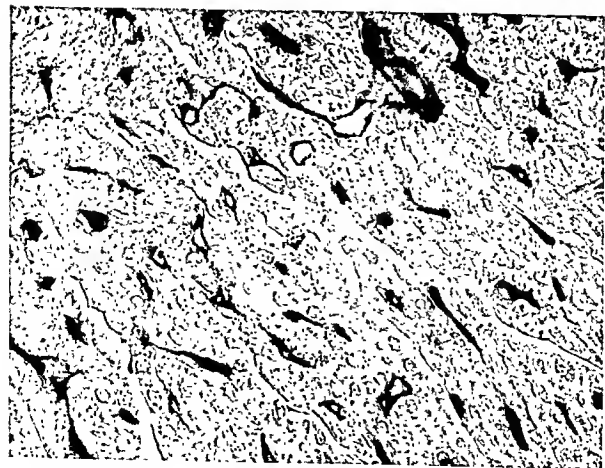


FIG. 1

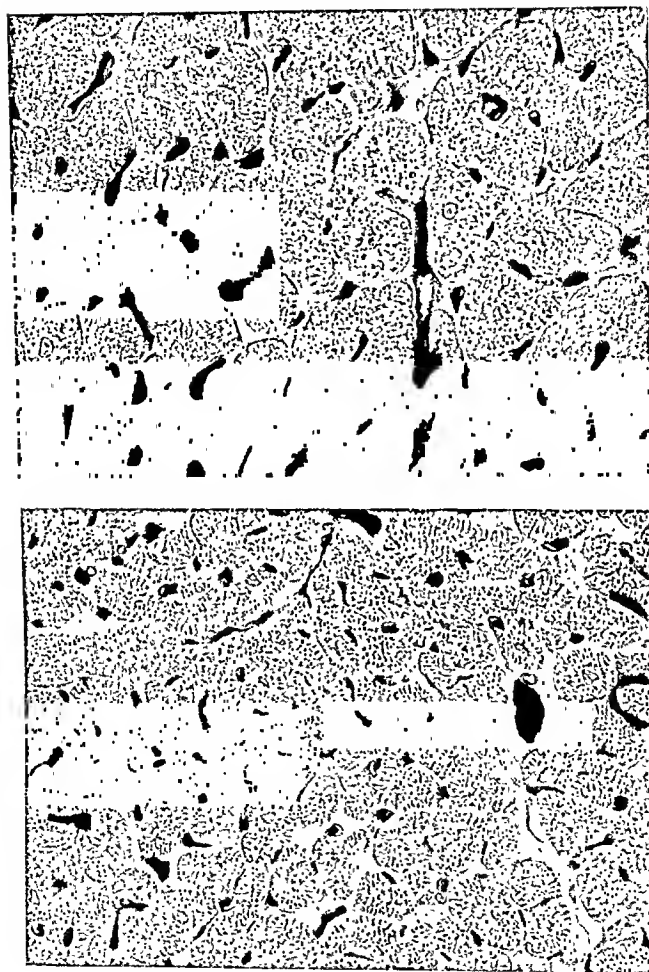


FIG. 3

(Shipley *et al.*, Capillary supply in hypertrophied hearts)

PRODUCTION OF HEMORRHAGIC NECROTIC SKIN
LESIONS IN THE RABBIT BY MEANS OF
HEMOPHILUS INFLUENZAE AND
HEMOPHILUS PERTUSSIS*

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The skin biological differentiation of *Hemophilus influenzae* and *Hemophilus pertussis* is known in the Japanese literature through the work of Takagi and Okutani, according to a report of Kasahara (1). In the European literature, Gundel and Schlueter (2) first called attention to a possible differentiation between *H. influenzae* and *H. pertussis* by means of the different reactions following inoculation into the skin of a rabbit.

H. influenzae, when injected intracutaneously in the abdominal wall of rabbits, causes inflammation and erythema. The color of this lesion is pinkish red; sometimes a tiny yellowish pustule develops in the center. *H. pertussis*, on the other hand, produces a bluish discoloration in the injected area which sometimes is transformed into a hemorrhagic necrotic lesion of indistinct outline within 2 to 3 days. Occasionally one or the other strain may display a somewhat different picture, especially if examined only once.

This report will describe the experimental conditions under which *H. influenzae* can be made to induce severe hemorrhagic necrotic lesions, and the differences in behavior between *H. pertussis* and *H. influenzae* in this respect.

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Material

The following strains of *H. pertussis* and *H. influenzae* were at our disposal:

Strains of H. pertussis

(a) Strain A ¹	(r) Strain H 56 ²
(b) " B ¹	(f) " R 59 ²
(c) " G 51 ²	(e) " L 62 ²
(d) " R 55 ²	(h) " S 63 ²

Strains of H. influenzae

(a) Strain 158 ¹	(r) Strain 3743—Eye culture ³
(b) " S 3 ¹	(f) " 3878—Sputum ¹
(c) " 2621—Postmortem lung puncture ³	(e) " 79—Sputum ³
(d) " 3607—Spinal fluid ²	(h) " 385—Chest fluid ³

For the cultivation of *H. pertussis*, a Bordet-Gengou medium was used. It was prepared in the following way.

500 gm. of peeled sliced potatoes are boiled until soft, in 1000 cc. of distilled water and 40 cc. of glycerine. The original amount of fluid is restored and strained through gauze. To 500 cc. of the filtrate, 1500 cc. of 0.6 per cent saline solution and 60 gm. of agar are added. After the agar has been dissolved by heat (avoid scorching), water is added to restore the original volume and the medium is bottled in 120 cc. amounts, autoclaved and refrigerated. If used, it is melted, cooled to 45°C. and 40 cc. of sterile, fresh, citrated or defibrinated rabbit blood warmed to 40–45°C. is added. Blood and medium are mixed gently without air bubble formation, poured into sterile culture tubes (5 x 5/8 inches), and allowed to congeal to slants. The surface should be smooth and moist, the color a rich cherry red. Since the pH is approximately 6.8, no adjustment of pH was necessary.

For the cultivation of *H. influenzae*, a Levinthal medium was used, prepared as follows:

2 per cent agar of pH 7.5 is melted and cooled to 60–70°C., and 5 to 10 per cent rabbit blood added. It is mixed well and put into a boiling water bath for 8 minutes if 1 litre is prepared; if 2 litres, for 12 minutes. Directly afterwards, the blood agar mixture is filtered through sterilized cotton which has been kept warm at about 60°C. The cotton may be moistened before sterilizing. Tubes (6 x 3/4 inches) are filled with the clear agar and allowed to congeal. All media are kept in the refrigerator until used. Chocolate slants also have sometimes been used for the cultivation of *H. influenzae*. 48 hour growth of *H. pertussis* and 24 or 48 hour growth of *H. influenzae* were used in the experiments.

¹ Obtained through the courtesy of the Department of Health, New York.

² Obtained through the courtesy of Dr. Schwartzman, The Mount Sinai Hospital, New York.

³ From the Bacteriological Laboratory of Beth Israel Hospital.

Suspensions of *H. pertussis* were obtained by taking off the growth from the Bordet-Gengou slants by means of a platinum loop or sterile swab, and put into a few cubic centimeters of normal saline. Suspensions of *H. influenzae* were obtained by adding 3 to 4 cc. of saline to a Levinthal slant. The growth on the surface was washed off by means of a platinum loop. The suspension was taken off with a sterile pipette and put into another Levinthal slant to increase the density of the bacterial suspension. Equal distribution was obtained by squeezing the suspension several times with a sterile pipette. We also had good results by taking off the bacterial growth from the Levinthal slant by means of a sterile swab and putting the swab into saline. The final bacterial content of the suspension (after being washed one to three times) used in our experiments, varied between 2 to 5 billion per cc.

For the preparation of *B. typhosus* agar washing filtrates, the technique of Schwartzman (3) has been employed.

For the preparation of agar washing filtrates of *H. influenzae*, *H. influenzae* has been cultivated on Levinthal-Kolle flasks which had been seeded with 3 to 4 cc. of a saline suspension of *H. influenzae* washed off Levinthal slants. The bacterial growth was washed off the Kolle flasks with 3 to 4 cc. of 0.85 per cent saline suspension with the addition of 0.4 per cent phenol. The suspensions were pooled after being controlled for purity, then centrifuged. The supernatant fluid was filtered through a Berkefeld V candle. The bacterial sediment was suspended in saline and washed three times.

Method

The rabbits (weighing between 4 and 5 pounds) were shaved on the abdomen and injected intradermally with 0.25 cc. of a bacterial suspension or bacterial filtrate. 24 hours later, 1 to 3 cc. of a bacterial suspension or filtrate were given intravenously. Special care has to be taken that the intradermal injection is really intradermal and not subcutaneous.

It must be mentioned here that in our experiments inflammation and infiltration without hemorrhagic necrotic changes were called negative, even if they were pronounced. Hemorrhagic necrotic lesions only were considered as positive. For the purpose of abbreviation the lesions are marked + to + + + +, according to the size of the lesion. Thus the difference between a negative and a one plus reaction is more important than the quantitative differences indicated by the number of plus signs. While it is very easy to read reactions caused by *H. influenzae*, readings of areas prepared with *H. pertussis* are somewhat more difficult. They show, almost from the beginning, a tendency to bluish violet discoloration which is followed by necrosis after a lapse of several days.

Influence of Intravenous Injection of Living H. influenzae on Areas Inoculated Intradermally with H. influenzae and H. pertussis

Experiment 1.—Two rabbits, 3-85 and 3-86, were injected intradermally with 0.25 cc. each of a suspension of

- (a) *H. pertussis* strain R 55 right upper quadrant
 (b) " " " H 56 " lower "
 (c) *H. influenzae* " 158 left upper "
 (d) " " " 2621 " lower "

24 hours later the reaction of the areas prepared with *H. pertussis* (a) and (b) read in both rabbits as follows: A central bluish spot is surrounded by a white zone which again is surrounded by bluish violet areas of indistinct outline. The

TABLE I

Production of Hemorrhagic Necrotic Lesions in Rabbit Skin Intradermally Injected with H. influenzae and H. pertussis by Means of Intravenous Reinjection of Living H. influenzae

Rabbit No.	Strain No.			
	R 55 <i>H. pertussis</i>	H 56 <i>H. pertussis</i>	158 <i>H. influenzae</i>	2621 <i>H. influenzae</i>
3-85	±	±	++++	++++
3-86	±	±	++++	+++

Read 5 hours after intravenous reinjection.

± = doubtful reaction.

+ to ++++ = different degrees of hemorrhagic necrotic lesions.

skin areas prepared with *H. influenzae* (c) and (d) show a quite different picture. They display erythema, infiltration and swelling; the color is pinkish red. There is a small pustule in the center.

The rabbits were now injected intravenously with 1.5 cc. of a suspension of living *H. influenzae* (about 3 billion organisms per cc.). The results obtained are shown in Table I.

It can be seen from Table I that the areas prepared intradermally with *H. influenzae* were transformed into hemorrhagic necrotic lesions a few hours following the intravenous injection. The intensity of the reaction in the area prepared with strain 158 was somewhat stronger in rabbit 3-86 than that with strain 2621. The aspect of the areas prepared intradermally with *H. pertussis* was scarcely, or not at all, changed.

During the observation time of 24 hours, the bluish violet discoloration of the skin areas prepared intradermally with *H. pertussis* increased in size and intensity.

The intravenous injection of *H. influenzae* had, however, no apparent influence upon the natural development of this lesion as proved in control rabbits which were not reinjected intravenously. In those rabbits, areas prepared with *H. influenzae* did not show any sign of hemorrhagic necrosis.

Influence of Intravenous Injection of Living H. pertussis on Areas Inoculated Intradermally with H. influenzae and H. pertussis

Experiment 2.—Three series, consisting of four rabbits each, were examined in the same order of experiment as in Experiment 1. But, instead of reinjecting intravenously suspensions of *H. influenzae*, suspensions of *H. pertussis* were given. In two of these series no reaction occurred whatsoever. In a third series in which double the amount of organisms were reinjected intravenously, hemorrhagic necrotic changes occurred in two out of four rabbits. Here, also, areas prepared intracutaneously with *H. influenzae*, only, became positive. There was no reaction in the areas prepared intradermally with *H. pertussis*.

Effectiveness of Heat-Killed H. influenzae upon Skin Areas Previously Inoculated with Different Strains of H. influenzae

Experiment 3.—0.25 cc. of a suspension of three strains of *H. influenzae*, 3607, 3743 and 3878, were injected intracutaneously into three different areas of the abdominal skin of three rabbits (2-29 to 2-31). 24 hours later, 1.5 cc. of a suspension of *H. influenzae*, strain 3607, was injected intravenously. The bacterial suspension employed for the intravenous reinjection had been put in a water bath at 60°C. for 1 hour. The fact that the bacilli had been killed by this procedure was controlled by culture. The results obtained are given in Table II.

Table II shows that the intravenous injection of heat-killed *H. influenzae* may also produce hemorrhagic necrotic lesions in skin areas of rabbits previously prepared intracutaneously with living *H. influenzae*. At the same time it can be seen that different rabbit individuals vary in their reactions towards different strains of *H. influenzae* if injected simultaneously. It cannot yet be decided whether or not the homologous strain has a somewhat more powerful reactivating potency.

Production of Hemorrhagic Necrotic Lesions in Skin Areas Injected Intradermally with Living and Killed H. influenzae by Means of Intravenous Reinjection of Living H. influenzae

Experiment 4.—0.25 cc. of a suspension of *H. influenzae*, strain 158, (a) living, (b) killed—in water bath, 1 hour, 60°C.; strain 3607 (c) living, (d) killed—in water

TABLE II

Production of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with Different Strains of H. influenzae by Means of Intravenous Reinjection of Heat-Killed H. influenzae

Strain No.	Before intravenous injection			3 hrs. after intravenous injection			24 hrs. after intravenous injection		
	Rabbit No.			Rabbit No.			Rabbit No.		
	2-29	2-30	2-31	2-39	2-30	2-31	2-29	2-30	2-31
(a) 3607, <i>H. influenzae</i>	—	—	—	++	++++	++	+	++	++
(b) 3743, <i>H. influenzae</i>	—	—	—	—	±	++	—	±	++
(c) 3878, <i>H. influenzae</i>	—	—	—	±	++++	—	—	++++	—

— = no hemorrhagic necrotic lesion.

± = doubtful reaction.

+ to ++++ = different degrees of hemorrhagic necrotic lesions.

TABLE III

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas Intradermally Prepared with Living and Killed H. Influenzae Following Intravenous Reinjection of a Suspension of Living H. influenzae

Strain No.	Before intravenous injection		3 hrs. after intravenous injection		5 hrs. after intravenous injection		24 hrs. after intravenous injection	
	Rabbit No.		Rabbit No.		Rabbit No.		Rabbit No.	
	2-39	2-40	2-39	2-40	2-39	2-40	2-39	2-40
158, <i>H. influenzae</i>								
(a) living.....	—	—	+++	+++	++++	++++	*	*
(b) dead.....	—	—	+	++	++++	++++	*	*
3607								
(a) living.....	—	—	+	++++	++++	++++	*	*
(b) dead.....	—	—	+++	+++	++++	++++	*	*

— = no hemorrhagic necrotic lesion.

+ to ++++ = different degrees of hemorrhagic necrotic lesions.

* Rabbit dead.

bath, 1 hour, 60°C., was injected intradermally into two rabbits (2-39 and 2-40). 24 hours later, 1.5 cc. of a suspension of living *H. influenzae*, strain 3607, was injected intravenously. The result of this experiment is given in Table III.

According to the experiment shown in Table III, the intravenous injection of living *H. influenzae* may induce hemorrhagic necrotic lesions in areas prepared 24 hours previously with living as well as with killed *H. influenzae*.

It may be added that the intravenous injection of heat-killed *H. influenzae* was able to elicit hemorrhagic necrotic lesions in areas prepared with killed *H. influenzae* also. We have the impression, however, that the intensity of the hemorrhagic lesions obtained under those conditions is weaker than that obtained with living bacilli.

*Influence of Intravenous Injection of B. typhosus Agar Washing Filtrate
on Skin Areas Prepared Intradermally with H. influenzae
and H. pertussis*

Experiment 5.—The following experiment was made to decide the question whether the intravenous injection of *B. typhosus* agar washing filtrate (Shwartzman toxin) can induce hemorrhagic necrotic lesions in the rabbit skin 24 hours previously prepared with *H. influenzae* and *H. pertussis*. For this purpose, 0.25 cc. of a suspension of living bacilli of

- (a) *H. pertussis* strain R 55
- (b) " " " H 56
- (c) *H. influenzae* " 158
- (d) " " " 2621 and

- (e) *B. typhosus* agar washing filtrate diluted 1:2

were injected intracutaneously into the skin of rabbit 3-87. 24 hours later, 1 cc. of a 1:3 dilution of *B. typhosus* agar washing filtrate was injected intravenously. The result of this experiment is summarized in Table IV.

As can be seen from Table IV, characteristic hemorrhagic necrotic lesions occurred in the areas prepared with *H. influenzae* as well as in the areas prepared as a positive control with *B. typhosus* agar washing filtrate following the intravenous injection of the latter. The areas prepared with *H. pertussis*, however, did not display any essential changes during the corresponding observation period.

Out of nine rabbits examined in this order, five gave identical or similar results. Three rabbits proved to be refractory, and one rabbit died shortly after the intravenous injection of *B. typhosus* agar washing filtrate.

Nineteen rabbits were injected intradermally with *B. typhosus* agar washing filtrate as well as with *H. influenzae*, and reinjected intravenously with *B. typhosus* agar washing filtrate. Of these rabbits, nine showed positive reactions in both areas and four were entirely negative. Six rabbits were positive in one area only, three of them in the area prepared with *H. influenzae*, but negative in the area prepared with *B. typhosus* agar washing filtrate; and conversely, three rabbits were positive in the area prepared with *B. typhosus* agar washing filtrate but negative in the area prepared with *H. influenzae*.

TABLE IV

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas of Rabbit 3-87 Previously Inoculated with H. influenzae and H. pertussis Following the Intravenous Injection of B. typhosus Agar Washing Filtrate

Strain No.	Before intravenous injection	3 hrs. after intravenous injection	7 hrs. after intravenous injection	24 hrs. after intravenous injection
(a) R 55, <i>H. pertussis</i>	±	±	+	+
(b) H 56, <i>H. pertussis</i>	±	±	+	+
(c) 158, <i>H. influenzae</i>	—	+++	++++	++++
(d) 2621, <i>H. influenzae</i>	—	++	+++	+++
(e) 1:2 <i>B. typhosus</i> agar washing filtrate.	—	++	++	++

— = no hemorrhagic necrotic lesion.

± = doubtful reaction.

+ to ++++ = different degrees of hemorrhagic necrotic lesions.

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with H. influenzae, as Well as with B. typhosus Agar Washing Filtrate, Following the Intravenous Reinjection of Living H. influenzae

Experiment 6.—To determine the effectiveness of the intravenous reinjection of living *H. influenzae* on areas prepared with *H. influenzae* as well as *B. typhosus* agar washing filtrate was the object of this experiment. Nine rabbits received intradermal injections of 0.25 cc. of suspensions of two to three different strains of *H. influenzae* and one intradermal injection of *B. typhosus* agar washing filtrate into the skin of the abdominal wall. 24 hours later, suspensions of living *H. influenzae* were given intravenously. Six of these rabbits developed marked hemorrhagic necrosis in one or more areas prepared with *H. influenzae* as well as with *B. typhosus* agar washing filtrate. The reactions obtained were of about the same strength. In one rabbit the lesion of the skin area prepared with *B. typhosus* agar washing filtrate was distinctly weaker than the area prepared with *H. influenzae*; in a second rabbit, just the opposite took place. One rabbit was refractory.

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with H. influenzae, as Well as with B. typhosus Agar Washing Filtrate, Following the Intravenous Reinjection of Killed H. influenzae

Experiment 7.—Eight rabbits have been examined in the same order as the preceding ones, the only difference being that the intravenous injection was performed with heat-killed *H. influenzae* (1 hour, 60°C.), instead of living ones. All eight animals reinjected intravenously with heat-killed *H. influenzae* developed hemorrhagic necrotic lesions in the areas prepared intracutaneously 24 hours previously with *B. typhosus* agar washing filtrate. Hemorrhagic necrotic lesions in areas prepared with living *H. influenzae* appeared in six out of the eight animals used.

TABLE V

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with Suspensions of Living H. influenzae and Their Corresponding Wash Waters Following the Intravenous Reinjection of B. typhosus Agar Washing Filtrate

Rabbit No.	Suspension of living <i>H. influenzae</i>		First wash water	Third wash water	<i>B. typhosus</i> agar washing filtrate
	Not washed	Washed three times			
5-63	+	+	—	—	—
5-64	++	++	—	—	++
5-65	++++	++++	±	—	++

Read 5 hours after intravenous reinjection.

— = no hemorrhagic necrotic lesion.

± = doubtful reaction.

+ to ++++ = different degrees of hemorrhagic necrotic lesions.

Comparison of the Effectiveness of Suspensions of Living H. influenzae with Their Corresponding Wash Waters

Experiment 8.—This experiment was made to decide whether the hemorrhagic necrotic lesions caused by *H. influenzae* are due to an exotoxin. Four rabbits, 5-62 to 5-65, were injected with 0.25 cc. of

- (a) Suspension of *H. influenzae* strain 3878 not washed
 (b) " " " " " " washed 3 times
 (c) First wash water of " " " " " "
 (d) Third " " " " " "

24 hours later the rabbits were reinjected with 1 cc. of *B. typhosus* agar washing filtrate to a dilution of 1:10. Rabbit 5-62 died soon after the intravenous reinjection. Table V shows the result in the remaining three rabbits.

It can be seen from Table V that the intravenous injection of *B. typhosus* agar washing filtrate induced hemorrhagic necrotic lesions in the areas prepared with the bacilli themselves, whether not washed at all or washed three times. In one rabbit, 5-65, a doubtful reaction developed in the skin area prepared with the first wash water, while the third wash water was entirely negative in all three rabbits. Berkefeld filtration was not applied in this experiment to avoid possible loss of reactivity through the procedure of filtration.

Comparison of the Effectiveness of Suspensions of Killed H. influenzae with Their Corresponding Wash Waters

Experiment 9.—This experiment was performed in the same order as the preceding one, but heat-killed suspensions of *H. influenzae* were used for the intradermal

TABLE VI

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with Suspensions of Heat-Killed H. influenzae and the Corresponding Wash Waters Following the Intravenous Reinjection of B. typhosus Agar Washing Filtrate

Rabbit No.	Suspension of killed <i>H. influenzae</i>		First wash water	Third wash water
	Not washed	Washed three times		
7-40	++	+++	—	—
7-41	++++	++++	+	—

Read 5 hours after intravenous reinjection.

— = no hemorrhagic necrotic lesion.

+ to ++++ = different degrees of hemorrhagic necrotic lesions.

injection instead of living ones. Three rabbits, 7-40 to 7-42, were injected with 0.25 cc. of

(a) Suspension of *H. influenzae* strain 79 killed, not washed

(b) " " " " " " " washed three times

(c) First wash water of suspension of *H. influenzae* strain 79 killed

(d) Third " " " " " " " " " "

24 hours later the rabbits were reinjected with 1 cc. of *B. typhosus* agar washing filtrate in a dilution of 1:10. Rabbit 7-42 died soon after this injection. The result in the remaining two rabbits is given in Table VI.

Hemorrhagic necrotic lesions occurred in both rabbits in the areas prepared with washed, as well as with unwashed bacilli. The wash waters proved to be completely ineffective in one rabbit, while the other showed a one plus reaction with the first wash water.

Examination of a Suspension of H. influenzae Cultured in Kolle Flasks and Its Corresponding Agar Washing Filtrate Produced According to the Technic of Schwartzman

Experiment 10.—Since Schwartzman and Frisch (4) obtained an effective agar washing filtrate from *H. influenzae*, Schwartzman's technic has been employed to compare their results with those obtained with the technic used in our experiments reported thus far. For this purpose, *H. influenzae* has been cultivated in Kolle flasks, and bacilli and wash waters examined. Three rabbits, 5-51 to 5-53, were injected intradermally with 0.25 cc. of

(a) Suspension of *H. influenzae* strain 3878

(b) First wash water of " " " " " "

(c) Berkefeld filtrate of (b) = *H. influenzae* Schwartzman toxin

24 hours later, the rabbits were reinjected intravenously with *H. influenzae* Berkefeld filtrate. Table VII shows the result of this experiment.

TABLE VII

Hemorrhagic Necrotic Lesions in Areas Prepared Intradermally with a Suspension of H. influenzae and the Corresponding Agar Washing Filtrate Following the Intravenous Reinjection of the Latter

Rabbit No.	Suspension of <i>H. influenzae</i>	First wash water	Berkefeld filtrate of first wash water
5-51	±	++++	++
5-52	—	—	—
5-53	—	—	—

Read 5 hours after intravenous reinjection.

— = no hemorrhagic necrotic lesion.

± = doubtful reaction.

+ to ++++ = different degrees of hemorrhagic necrotic lesions.

As can be seen from Table VII, an effective agar washing filtrate was obtained from *H. influenzae* grown in Kolle flasks. The intravenous reinjection of this filtrate induced hemorrhagic necrosis in one rabbit in the area prepared with both first wash water and its Berkefeld filtrate, while the area prepared with the bacilli gave only a doubtful reaction. The reaction in the area prepared with *H. influenzae* Berkefeld filtrate is somewhat weaker than that in the area prepared with the first wash water, probably due to a loss of reactivity through the process of filtration.

Comparison of the Influence of the Technic Employed in Cultivation of H. influenzae on the Occurrence of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with Suspensions of the Bacilli and Their Corresponding Berkefeld Filtrates

Experiment 11.—This experiment deals with the comparative study of *H. influenzae* when cultured on slants (inoculated with platinum loop) and in Kolle flasks (inoculated with several cubic centimeters of suspensions of *H. influenzae* in

TABLE VIII

Comparison of the Influence of the Technic Employed in Cultivation, on the Occurrence of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with H. Influenzae and Their Corresponding Berkefeld Filtrates, Following the Intravenous Reinjection of Suspensions of H. Influenzae and Their Berkefeld Filtrates

Rabbit No.	From Kolle flasks			From slants		<i>B. typhosus</i> agar washing filtrate
	Suspension of <i>H. influenzae</i>	Berkefeld filtrate of first wash water	Berkefeld filtrate of third wash water	Suspension of <i>H. influenzae</i>	Berkefeld filtrate of first wash water	
6-08	++	+++	±	+	±	++++
6-09	+++	+	—	++	—	++++
6-10	+++	+++	—	+++	—	++++
6-11	±	—	—	±	—	+++
6-12	—	—	—	—	—	+++
6-13	—	—	—	—	—	—

Read 5 hours after intravenous reinjection.

— = no hemorrhagic necrotic lesion.

± = doubtful reaction.

+ to ++++ = different degrees of hemorrhagic necrotic lesions.

saline). Six different areas of the abdominal skin of rabbits 6-08 to 6-13 were prepared intradermally with 0.25 cc. of

- | | | |
|-----|---|---------------------------------|
| (a) | Suspension of <i>H. influenzae</i> strain 385 | } Obtained from
Kolle flasks |
| (b) | Berkefeld filtrate of " " " " " " | |
| (c) | " " from third washing " " " " | |
| (d) | Suspension of <i>H. influenzae</i> " " | } Obtained from
agar slants |
| (e) | Berkefeld filtrate of " " " " " " | |
| (f) | <i>B. typhosus</i> agar washing filtrate | |

24 hours later they were divided into two series of three rabbits each. The first series was reinjected intravenously with suspensions of *H. influenzae* grown in Kolle flasks and washed three times; the second series with the first Berkefeld filtrate (b). The result of this experiment is summarized in Table VIII.

Table VIII shows that all three rabbits reinjected with suspensions of *H. influenzae* displayed hemorrhagic necrotic lesions in the areas prepared with *H. influenzae* from Kolle flasks as well as from agar slants. The Berkefeld filtrate prepared from growths in Kolle flasks (b) also gave a positive reaction, in contrast to the Berkefeld filtrate prepared from *H. influenzae* grown on agar slants (e). The Berkefeld filtrate prepared from the third washing gave a doubtful reaction in one rabbit only. Nevertheless, the washed bacilli, if injected intravenously, were able to produce hemorrhagic necrotic lesions. The three rabbits of the second series, reinjected with *H. influenzae* Berkefeld filtrate containing skin-sensitizing properties, did not induce any positive reactions in this experiment, except in the control areas prepared with *B. typhosus* agar washing filtrate. One rabbit showed a doubtful reaction in the area prepared with the bacilli themselves.

DISCUSSION

The experiments reported in this paper point to further differences in the skin biological behavior of *H. influenzae* and *H. pertussis*. They show that, under certain experimental conditions, it is possible to induce severe hemorrhagic necrotic lesions in the rabbit skin by means of *H. influenzae*. Preliminary experiments seem to prove the significance of the reported order of experiment for studies on involvement of the lung, if the first injection of *H. influenzae* is applied intranasally instead of intradermally. Shope demonstrated the rôle of *H. influenzae* in the pathogenesis of swine influenzae particularly. He showed that *H. influenzae* is able to activate the "virus disease" in swine. Concerning the production of hemorrhagic necrotic lesions in the skin of rabbits, the experiments of Schwartzman and his co-workers (5, 6) are of special interest. According to these authors, it is possible to obtain effective exotoxins from a large number of microorganisms, including *H. influenzae* and *H. pertussis*. We, too, could obtain a moderately effective exotoxin from *H. influenzae*, provided Schwartzman's method of cultivation was used. As far as it is possible to draw any conclusions from the experiments reported, it seems to be unlikely that exotoxins are a decisive factor in the pathogenesis of hemorrhagic necrotic lesions caused by *H. influenzae* under the given experimental conditions. This statement is upheld by the ability of

washed living and heat-killed *H. influenzae* to exhibit reactions, and the lack of this ability of the supernatant fluids and wash waters. The special attention given to the production of exotoxins and their undoubted importance may be the reason why factors have been overlooked so far, which may play an important rôle in the course of bacterial infections as such.

CONCLUSIONS

1. The intradermal injection of *H. influenzae* in the abdominal wall of rabbits induces inflammation, frequently combined with a central pustule. The corresponding injection of *H. pertussis* causes a bluish violet discoloration of the skin area involved which undergoes slight hemorrhagic necrotic changes within a few days.

2. The intravenous injection of living *H. influenzae*, 24 hours after the intradermal inoculation with living *H. influenzae*, is able to transform the respective skin areas into severe hemorrhagic necrotic lesions within 3 to 5 hours.

3. Heat-killed *H. influenzae*, if injected intravenously, may produce hemorrhagic-necrotic lesions in areas previously prepared with living or heat-killed *H. influenzae*.

4. *H. pertussis*, if injected intravenously, may cause, perhaps to a lesser extent, hemorrhagic necrotic lesions in skin areas 24 hours previously injected with *H. influenzae*.

5. The normal course of the infection of rabbit skin with *H. pertussis* is not, or not essentially, influenced by intravenous reinjection of living or killed *H. influenzae* or *H. pertussis*.

6. The agar washing filtrate of *B. typhosus*, if injected intravenously, can produce hemorrhagic necrotic lesions in rabbit skin prepared intracutaneously with living as well as with heat-killed *H. influenzae*. The intravenous injection of *B. typhosus* agar washing filtrate has no influence on areas prepared with *H. pertussis*.

7. Conversely, *H. influenzae* as well as *H. pertussis*, if injected intravenously, are able to produce hemorrhagic necrotic lesions in rabbit skin prepared 24 hours previously with *B. typhosus* agar washing filtrate.

8. The effectiveness of suspensions of *H. influenzae* apparently is confined to the bacteria themselves rather than to the supernatant

fluids. This does not exclude the possibility of producing effective exotoxins under special experimental conditions.

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STUDIES ON THE SOMATIC C POLYSACCHARIDE OF PNEUMOCOCCUS

I. CUTANEOUS AND SEROLOGICAL REACTIONS IN PNEUMONIA

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In 1930, Tillett and Francis (1) observed that the sera of individuals acutely ill with lobar pneumonia possess the capacity of precipitating a non-protein, carbohydrate fraction derived from *Pneumococcus*. This fraction was termed the C substance. It was shown that the precipitating action of patients' serum with the C substance is demonstrable early in the course of pneumonia, persists throughout the course of the active disease, and disappears during convalescence.

Subsequent tests with sera from certain other diseases showed that the precipitation of fraction C is not limited to the sera of individuals with pneumococcus infection. The phenomenon was also demonstrated in cases of rheumatic fever, bacterial endocarditis, and lung abscess, but not in all febrile diseases. The sera of patients suffering from malaria, tuberculosis of the lungs, typhoid fever, measles, and varicella did not react with fraction C. While the exact nature of the mechanism of the precipitation reaction with C is not understood, Tillett and Francis suggested that the principles involved in the anamnestic reaction described by Cole (2) might be used to explain the phenomenon.

Further studies by Tillett, Goebel, and Avery (3) on the chemical and immunological properties of the C substance showed that it is a complex polysaccharide contained within the body of the pneumococcus cell. It is species specific and is found in all pneumococci irrespective of type or virulence. It is distinct from the type specific capsular polysaccharide not only in its chemical properties but in its serological reactivity. Later, Heidelberger and Kendall (4) showed, in a study of the chemical properties of the polysaccharides of Type

IV Pneumococcus, that the C substance contains 4 per cent of phosphorus firmly bound in organic combination.

A preliminary report on the cutaneous reactions to C in 30 cases of pneumonia has previously been given (5). Recently amounts of the somatic polysaccharide of sufficient purity have been obtained to undertake a further study of both the cutaneous and serological reactions in pneumonia to this substance. Data included in the present paper record primarily the relationship of the cutaneous reaction to the clinical course and outcome of the disease, and the parallelism between the *in vitro* serum precipitation reaction and the *in vivo* response to intradermal injection of C polysaccharide. Observations on the nature of the C precipitation reaction have been reserved for subsequent publication.

Materials and Methods

Subjects.—The patients observed in this study were admitted to the wards of the Hospital of The Rockefeller Institute. Group A comprised 46 cases of pneumococcus lobar pneumonia, of which 39 recovered and 7 died. As soon as possible after entry sputum typing was attempted by means of the Neufeld reaction. This was successful in a high proportion of the cases. When unsatisfactory, however, the typing was determined by the mouse method. Distribution of types among the 46 cases was as follows: Type I, 11 cases; Type II, 2 cases; Type III, 9 cases; Type V, 1 case; Type VII, 4 cases; Type VIII, 6 cases; Type IX, 2 cases; Type X, XIV, XVI, XVIII, 1 case each; and unclassified types, 7 cases. On admission, a blood culture, complete blood count, and roentgenogram of the chest were made on each patient. The progress of the pneumonic lesion was followed by daily physical examination of the chest and by frequent x-ray examination throughout the course of the disease. The general treatment of each patient was the same. Specific serum treatment was given to all but one patient with Type I infection, to a smaller number of individuals with Type II, and to a few patients with Type V, VII, and VIII infection. Artificial pneumothorax was used in a few instances.

Group B comprised 10 patients suffering from diseases other than pneumococcus pneumonia. There were 5 patients in whom the diagnosis of rheumatic fever had been made; 3 patients ill with bacterial infections of known etiology; and finally 2 cases of non-infectious fevers.

Group C comprised 19 normal healthy adults free from any obvious infection. None of these individuals had had pneumonia.

Skin Testing Materials.—The C substance used for intracutaneous inoculation was prepared in a state of high purity by methods employed in this laboratory (3) from an R strain derived from Type II pneumococci. The preparation was a

white powder, readily soluble in physiological salt solution. Intracutaneous injections of a solution of C substance in amounts of 0.1 cc. containing 0.1 mg. of polysaccharide were given to all the patients. Physiological salt solution, 0.1 cc., was used as a control. Frequent skin tests were performed on the patients ill with lobar pneumonia (group A). An initial test was always performed shortly after the admission of each patient, and was repeated at intervals during the course of the disease. Tests on the control cases (groups B and C) were made less frequently. The inoculated sites were observed frequently during the succeeding 24 hours.

Precipitation Tests.—Serum was collected before each intradermal inoculation and the samples were tested for capacity to precipitate in the presence of the C substance. The precipitation tests were performed in the usual manner, *i.e.*, 0.2 cc. serum, diluted with 0.3 cc. of physiological salt solution was mixed with 0.5 cc. of varying dilutions of the C substance. Readings were made after the mixtures had been incubated at 37°C. for 2 hours and had stood in the ice box overnight. In positive tests the precipitate that forms settles rapidly to the bottom of the tubes, and on shaking can be readily broken up. No disc formation is seen.

The Cutaneous Reaction

The capacity of two other components of the pneumococcus cell, the type specific capsular polysaccharide and the nucleoprotein fraction, to elicit characteristic cutaneous reactions in pneumonia has been described previously (6). The reaction to the capsular polysaccharide is of the immediate wheal and erythema variety, is type specific, and appears only when the recovery processes have been initiated. It may persist during convalescence and is always associated with the presence of type specific antibodies in the patient's serum. The skin reaction to the nucleoprotein, on the other hand, is of the delayed variety, not appearing until several hours after inoculation, is negative during the acute stages of pneumonia, and becomes positive late in the period of convalescence. A high percentage of normal individuals react to the bacterial protein injected intradermally. Circulating antibodies to the nucleoprotein, as measured by precipitin tests, are demonstrable in about equal titer both during the acute disease and the period of recovery.

In striking contrast to the cutaneous reactions elicited with the capsular polysaccharide which occur with the onset of recovery, and those following the injection of the nucleoprotein which appear later in convalescence, cutaneous reactions to the C substance are present

during the height of the disease. The character of the positive cutaneous reaction is distinctive and may be described as follows:

Within 15 to 30 minutes following the intradermal inoculation of 0.1 mg. of the C substance there appears at the site of injection a wheal surrounded by a zone of erythema. This reaction in its early development resembles the capsular polysaccharide skin test, but the zone of erythema is less intense and "pseudopods" extending out from the central wheal, so frequently seen in the latter, are rarely encountered. Within an hour this acute phase has usually faded and is then followed by the delayed reaction, an edematous erythema. The center of the area of delayed erythema is frequently dark, reddish brown, sometimes hemorrhagic, about which there may be a pale white halo, and beyond this a bright red erythema. The delayed reaction begins to appear in 2 to 3 hours, is well marked in 6 to 10 hours, persists for 18 to 24 hours, and then fades, leaving a residual brown stain. The size of reacting area may reach 5 cm. in diameter, but is usually 2 to 3 cm. in maximum diameter. There is frequently tenderness at the site of injection. The skin test is considered positive if a delayed reaction occurs with an area of erythema larger than 1 cm. in diameter. The reaction is called doubtful if the erythema is between 5 and 10 mm., and negative if the erythema is less than 5 x 5 mm. While the classification is an arbitrary one, experience has justified the use of this relative standard.

Results in Pneumococcus Pneumonia

Recovered Cases.—Frequent observations upon the C skin test in 39 recovered cases of pneumococcus pneumonia have shown that in all patients, irrespective of the type of invading pneumococcus, a positive reaction was elicited during the acute stage of the disease. The reaction appeared early, in one instance as early as 12 hours after the initial chill, and persisted throughout the acute febrile period. With the onset of recovery, whether associated with crisis or lysis, the reaction decreased markedly in intensity, and during an uncomplicated convalescence, in the majority of cases could no longer be demonstrated. Parallel determinations of the serum and cutaneous reactions with the C substance have shown striking uniformity in results and have confirmed the observations of Tillett and Francis on the appearance of the precipitation reaction during the acute phase and its disappearance in convalescence. Chart 1 depicts graphically the occurrence of the cutaneous and serological reactions to the C substance in a typical uncomplicated recovered case of pneumococcus pneumonia.

The patient was a 21 year old mechanic admitted to the hospital on the second day following a characteristic onset of acute lobar pneumonia. The admission physical examination revealed consolidation of the lower portion of the right upper lobe which was confirmed by x-ray. Type XIX Pneumococcus was recovered from the sputum. Blood culture was sterile. The lesion spread to involve the whole of the right upper lobe. A crisis occurred on the 6th day of the disease, after which an uncomplicated convalescence followed. He was discharged on the 21st day, completely recovered.

A skin test with 0.1 mg. pneumococcus C fraction on the day of admission showed a strongly positive reaction which first appeared 3 hours after inoculation

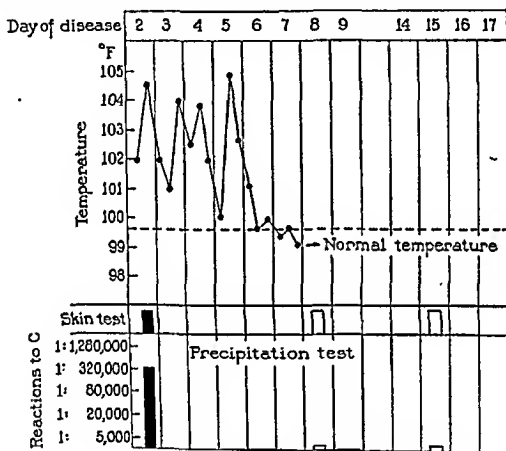


CHART 1. Type XIX pneumococcus pneumonia.

as a reddish brown area of erythema 1.1 x 1.3 cm. in size. This lesion gradually increased in size, becoming of maximum intensity at the end of 24 hours. The center was a deep red color surrounded by a thin white halo, around which an area of erythema 2 x 2.5 cm. was present. Subcutaneous edema was noted, and the lesion was slightly tender. A control injection of normal saline done at the same time was negative. Serum taken on the day of admission precipitated the C substance to a dilution of 1:320,000. Following the crisis skin tests were repeated, on the 8th and 15th days of the disease. On neither occasion was the skin reaction positive to 0.1 mg. of the C substance. At the same time, precipitation tests with the patient's serum were also negative.

Persistence of the positive cutaneous and serum reactions to the C substance was frequently noted in patients in whom the disease ran a protracted course by reason of delayed resolution or the occurrence of some complication, such as sterile pleural effusion or empyema. With the onset of convalescence, however, both reactions to the C substance disappeared. Moreover, reappearance of the C reaction was noted in a few patients who suffered some complication late in the disease after the acute febrile period had subsided. The following observa-

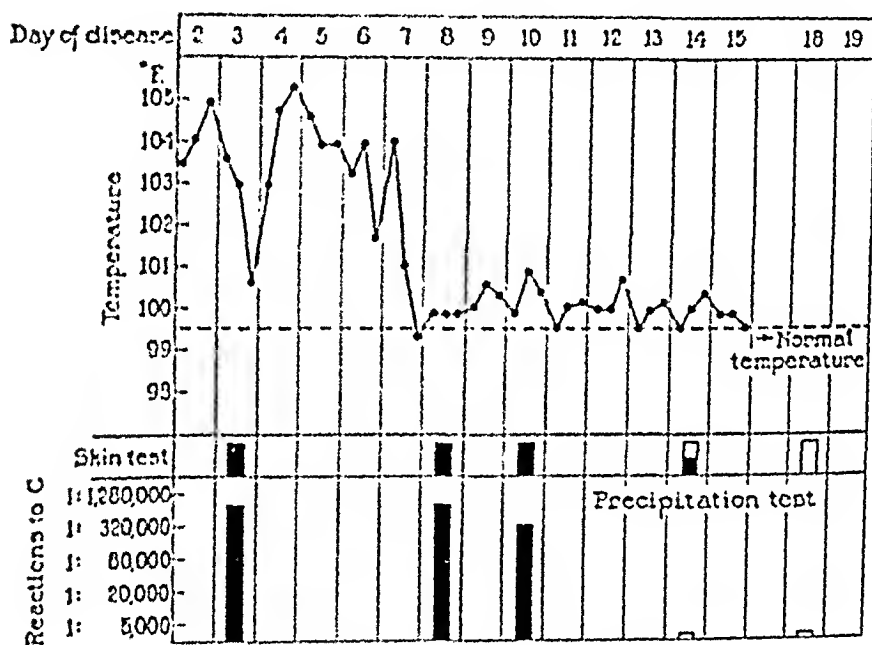


CHART 2. Type V pneumococcus pneumonia.

tions were made upon an individual in whom the disease was complicated by the development of pleural effusion, and the results of the serum and skin tests are represented in Chart 2.

The patient was a 39 year old white male admitted to the hospital 24 hours after an acute onset of pneumonia with chill, cough, and pain in the lower left chest. Physical examination revealed consolidation of the left lower lobe. Pneumococcus Type V was recovered from the peritoneal cavity of sputum-injected mice. In order to relieve the intense pleural pain artificial pneumothorax was attempted, but upon entering the pleural cavity a small amount of thick turbid fluid was dis-

covered, and consequently no air was injected. Culture of the pleural fluid yielded *Pneumococcus* Type V. On the 4th day of disease, an extension of the pneumonia to the left upper lobe occurred. On the 5th day a small amount of fluid, which proved to be sterile on culture, was removed from the left chest. Subsequently additional fluid accumulated, and on the 7th, 13th, and 16th days of his disease a total of 770 cc. of sterile fluid was aspirated. Following a crisis on the 7th day there was marked improvement in his general condition, although the temperature did not remain permanently normal until the 16th day. He was discharged completely recovered on the 33rd day.

Both serum and skin tests with the C substance were done on the 3rd day of acute illness and repeatedly following crisis. The initial skin test was markedly positive, and at the same time the patient's serum precipitated the C substance in dilution of 1:640,000. On the day following the crisis, at a time when the skin reaction in uncomplicated cases is usually negative or at most weakly positive, a strongly positive reaction was observed, and the precipitability of the patient's serum with C substance was still marked. These findings were confirmed 2 days later, at which time a large collection of pleural fluid was present. On the 14th day a doubtfully positive skin reaction, an area of erythema between 5 and 10 mm. in diameter, was observed, but the precipitation test was negative. During the convalescent period neither the skin nor serum reaction to C could be demonstrated.

Fatal Cases.—While in the recovered cases positive cutaneous and serological reactions were observed throughout the acute phase of the disease, persisting or reappearing if complications developed, and disappearing with the onset of recovery, in the fatal cases sharp differences were noted in the skin reactivity to C. Seven of the 46 cases terminated fatally. Three of these patients had Type III pneumococcus infections; in the remaining four patients pneumococci of Types I, II, VII, and X were the invading organisms. All seven of these patients failed to give a skin reaction to the C substance during some stage of the acute disease. Two of the seven cases gave positive skin reactions upon admission, but subsequent tests later on in the acute phase of the disease were negative. Parallel observations on the serum of these patients, however, showed that precipitation with the C substance occurred in samples tested throughout the disease. Chart 3 presents the findings in one patient illustrative of five fatal cases in each of which skin reactions were uniformly negative, although the serum reacted positively during the entire course of disease.

The patient was a 45 year old school teacher admitted on the 1st day of illness. His past history revealed a tuberculous infection of the lungs in 1932 which by

x-ray had subsequently been shown to be inactive. The onset of the present illness was abrupt, with the classical chill, pain in the left chest, and cough with bloody sputum. He was acutely ill when first seen, and examination disclosed an extensive consolidation of the left lower lobe. Type III *Pneumococcus* was identified in the sputum. Because of the marked pleural pain attempts were made on two occasions to induce a small artificial pneumothorax, but these proved unsuccessful because of multiple pleural adhesions. The patient became progressively worse with the spread of the pneumonic consolidation to involve all pulmonary lobes. Blood cultures remained consistently negative. Death occurred on the 6th day of disease.

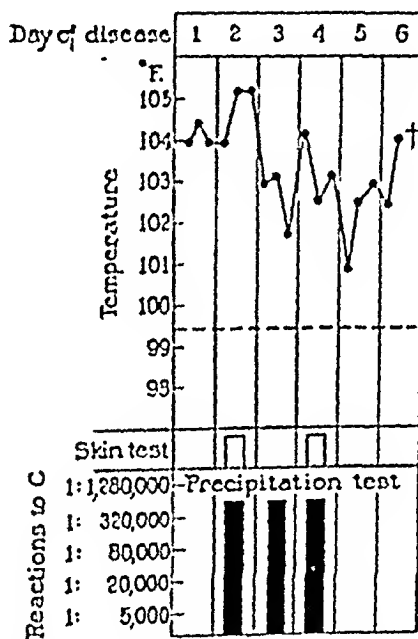


CHART 3. Type III pneumococcus pneumonia.

Skin reactions to the C substance on the 2nd and 4th days of disease were negative. However, precipitation of C by samples of serum taken on the 2nd, 3rd, and 4th days of disease occurred in high titer.

A loss of skin reactivity to C substance occurred during the course of the acute disease in two of the seven fatal cases. In both instances the skin reaction was positive on admission, but subsequent tests failed to provoke cutaneous reactions, although the serum throughout the disease precipitated when C was added in high dilution. A case illustrative of these findings is given in Chart 4.

The patient was a 37 year old white male admitted on the 5th day of lobar pneumonia of classical onset. Examination showed involvement of the right middle and lower lobes. Type X *Pneumococcus* and *Hemophilus influenzae* were recovered from the sputum. The admission blood culture showed no growth, but on the 8th day a culture was positive, less than 1 colony per 2 cc. of blood, and on the 12th day the colonies had increased to 50 per cc. of blood. The right upper and left upper lobes became successively involved and with this spread the signs of toxemia increased. He finally succumbed on the 13th day of disease despite the usual supportive measures. Postmortem examination showed extensive

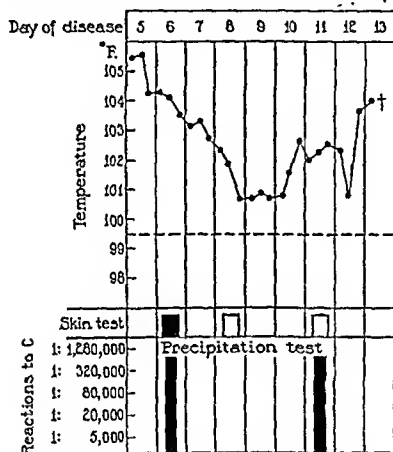


CHART 4. Type X pneumococcus pneumonia.

consolidation of the right upper, right middle, and left upper lobes, and beginning resolution in the right lower lobe.

A skin test done on the 6th day showed a markedly positive reaction, similar to that seen in the acute stage of cases which subsequently recover. There was an area of erythema 2 cm. in diameter, and at the same time the serum was precipitated in the presence of dilution of C as high as 1:640,000. However, on the 8th day, 5 days before death, no skin reaction to C substance was elicited, and none when the test was repeated on the 11th day of disease. Unfortunately, the serum precipitation test was not done on the 8th day, but on the 11th day it was found to be markedly positive.

A summary of results obtained in 46 cases of pneumococcus pneumonia is given in Table I.

Results in Control Cases

In the light of the observations of Tillett and Francis (1) and of Ash (7), who found that precipitation with C was not confined to the sera of individuals with pneumococcus pneumonia, it seemed of interest to test the C skin reactions in a group of patients suffering from other febrile diseases. For this purpose ten individuals were selected for study. The fever in 8 of the patients was known to be of infectious origin. In this group were some types of infection in

TABLE I
*Cutaneous and Serological Reactions to C Polysaccharide in 46 Cases of
Pneumococcus Pneumonia*

Classification	No. of cases	Stage of disease	Skin reactions			Precipitation tests	
			Positive	Doubtful	Negative	Positive	Negative
Recovered	39	Acute	39	0	0	39	0
		Convalescent	2	1	36	1*	38
Fatal	7	Acute	0†	0	7	7	0

* Convalescence complicated by active rheumatoid arthritis.

† 2 of 7 fatal cases gave positive reactions when first admitted. Reaction subsequently became negative.

which the serum precipitation test with C had previously been noted by the above mentioned authors. In the remaining 2 cases the fever was found to be of non-infectious origin. As a further control cutaneous and serological reactions were noted in a group of normal healthy adults.

Infections Fevers.—Observations were made upon 5 individuals with acute rheumatic fever. Each of these patients was tested during the active febrile period and again some weeks later when clinically and by laboratory tests the disease was considered to be quiescent. In all 5 patients the cutaneous reaction to the C substance was positive during the febrile period and became negative during the inactive phase of the disease. Precipitation tests with the serum of these individuals paralleled the cutaneous reaction, except in one case in which the serum failed to precipitate with C during the acute phase of the disease; the serum

of all 5, however, showed an absence of precipitation reaction with C during the convalescent period.

In addition to the 5 cases of rheumatic fever, observations were made upon an individual with subacute bacterial endocarditis. On admission to the hospital the patient was found to have chronic rheumatic heart disease and septicemia due to *Streptococcus viridans*. His course was steadily downward, with progressive toxemia and multiple embolic phenomena, finally terminating 1 month later with a pulmonary embolus. The skin reactions to C were markedly positive both when tested 1 week after admission and 9 days before death. At the same time the patient's serum precipitated C when the substance was added in high dilution.

One patient with empyema of hemolytic streptococcus origin was similarly studied. When tested twice during the acute febrile disease the cutaneous and

TABLE II

Cutaneous and Serological Reactions to C Polysaccharide in 29 Control Cases

Diagnosis	No. of cases	Stage of disease	Skin reactions		Precipitation tests	
			Posi- tive	Nega- tive	Posi- tive	Nega- tive
Rheumatic fever	5	{ Active Inactive	5 0	0 5	4 0	1 5
Bacterial endocarditis	1	Acute	1	0	1	0
Empyema-hemolytic Streptococcus	1	Acute	1	0	1	0
Aplastic anemia and <i>B. coli</i> septicemia	1	Acute (shortly before death)	0	1	1	0
Myeloblastic leukemia	1	Acute	0	1	0	1
Malaria	1	During paroxysm	0	1	0	1
Normal individuals	19	—	1	18	0	19

serum reactions with the C substance were strongly positive. Tests during convalescence could not be done because the patient was transferred to another hospital for operation.

Finally, tests were made upon one patient with aplastic anemia whose disease terminated in the development of septicemia with *Bacillus coli*. A skin test with C substance performed 4 days before death, at which time the blood had not yet been invaded, was completely negative. The serum, however, precipitated when C was added in dilution as high as 1:640,000. It will be noted that the results of the skin and serum tests in this case were precisely the same as those in the fatal cases of pneumococcus pneumonia. It was of interest to note at autopsy of this patient that, in addition to an aplastic bone marrow, hemochromatosis, and abscesses of the kidneys, a bronchopneumonia was present.

Non-Infectious Fevers.—Observations made on 2 cases of non-infectious fevers

showed negative cutaneous and serum reactions to the C substance. One patient with acute myeloblastic leukemia was tested on two different occasions, but at neither time reacted positively. The second patient, admitted for treatment of chronic ameloblastoma, developed a recurrence of a latent malarial infection while in the hospital. The parasites of tertian malaria were found in the blood, and a series of four chills with rises in temperature to 104-105° occurred prior to the administration of quinine. The skin and serological tests done at the height of one of these febrile paroxysms were completely negative.

Normal Individuals.—In order to determine the reaction of normal individuals to C, a group of 19 healthy adults free from obvious infection were selected for study. Skin and precipitation tests were performed in the manner described above. The serum of these individuals did not precipitate with the C substance even when the latter was added in high concentration, and the skin tests were negative in all but one instance. This person, upon subsequent inquiry, gave a history of hay fever and chronic postnasal discharge for many years and was known to be skin-sensitive to a variety of bacterial antigens as well as to pollen. Whether or not the positive reaction to C substance in this individual was related to a widespread sensitivity cannot be stated. A summary of results in the control cases is given in Table II.

DISCUSSION

From the present study of 46 patients with pneumococcus pneumonia it is apparent that the presence or absence of the cutaneous reactions to the C substance bears a definite relationship to the clinical course and outcome of the infection. In the 39 recovered cases all patients gave a positive skin reaction to the C substance during the acute illness. In a few patients in whom the disease was prolonged by complications the skin test remained positive until recovery ensued. Although in certain cases the capacity of the skin to react did not disappear abruptly, the test became negative in all but three patients within the first few days of convalescence. In striking contrast to these results are the findings noted in seven fatal cases. All of the latter patients failed to react to the C substance during the acute stage of the disease. Of particular interest were the results of skin tests in 2 fatal cases in which the reactions were positive early in the disease and later became negative as the disease progressed.

Simultaneous observations on the precipitability of the serum with C have confirmed and extended the observations of Tillett and Francis. In the recovered group, as well as in the fatal cases, sera taken during the febrile period precipitated on the addition of minute amounts of C

substance. Moreover, the precipitation test was positive extremely early in the disease. The serum remained positive if complications ensued, but became negative when normal convalescence followed.

The failure of the skin to react to the C substance despite the presence of a positive serum precipitation test would appear to be of prognostic significance. However, no conclusions can be made regarding the importance of the test as a prognostic sign until a larger group of cases has been studied. Tillett and Francis in their original paper noted that the precipitation test with C was not specific for pneumococcus infection. In a small group of cases they observed that "precipitation of C fraction occurred most definitely in those instances where Gram-positive cocci were proven to be or were suspected of being the etiological agent." These implications, however, were not substantiated by the work of Ash (7), who found that the serum of certain patients ill with Gram-negative bacillus infections possess the capacity of precipitating C. These observations, therefore, together with the results of studies now being carried out in this laboratory, to be reported later, suggest that the C precipitation phenomenon differs from usual immune reactions.

It is apparent, from the present investigation of 46 cases of pneumonia, and of 29 control patients, that the results of the skin and serum tests with C roughly parallel each other. Only in the fatal cases were the observations at variance. Thus, in view of this parallelism and despite the non-specific character of both reactions it would seem that some factor or change occurring in the serum in response to bacterial infection is capable of being mobilized in the tissues and thereby of reacting locally with the C substance. That some factor or change in the serum is essential for the skin reaction is suggested by the fact that the cutaneous response is almost invariably accompanied by a positive serum test. However, that the serum factor alone is solely responsible for the skin reaction seems improbable in the light of results in the fatal cases, in which the cutaneous test was negative although the serum reacted positively in precipitation tests. It would seem, therefore, that in addition to the serum factor, the state of reactivity of the tissue cells is also essential for the cutaneous response to C. When tissue reactivity is depressed by toxic products of the disease, as seems not improbable in fatal cases, the skin

test may be negative even though the serum reacts with C *in vitro*. A similar hypothesis has been advanced by Francis (8) in an explanation of the failure to obtain skin reactions to the capsular polysaccharide in fatal cases of pneumococcus pneumonia.

A recent communication by Finland and Dowling (9) has confirmed our earlier report upon the delayed cutaneous reactions to the somatic C polysaccharide. Using preparations of the "cellular carbohydrate" of Wadsworth and Brown (10) these authors found that "delayed cutaneous reactions with the 'cellular carbohydrate' of an atypical Type I Pneumococcus were obtained regularly during the febrile stage of a variety of infectious diseases and could not be elicited soon after recovery in such cases." Wadsworth and Brown in their original publication reported that the cellular carbohydrates were distinct from the soluble specific substance (SSS) and also from the C fraction, and gave type specific reactions with immune horse serum. Our experience with preparations of the cellular carbohydrate¹ has been limited to a few observations on the precipitation test with acute phase pneumonia sera. The sera of patients during the height of the disease have been found to precipitate with each preparation of the cellular carbohydrate tested, irrespective of the specific type of Pneumococcus from which the material was isolated. While the titer of these precipitation reactions was not so high as with the C substance, the tests were in each instance markedly positive. These observations, if confirmed in a larger series of cases, would indicate that the cellular carbohydrate of Wadsworth and Brown irrespective of specific type derivation may react with human sera during the acute stage of pneumonia as does the C substance.

SUMMARY AND CONCLUSIONS

A study of 46 cases of pneumococcus pneumonia has shown that a characteristic response may be elicited by the intracutaneous injection of 0.1 mg. of the somatic C polysaccharide of pneumococcus. During the acute febrile period in patients who recover, the response consists of a delayed erythema which reaches its maximum intensity in 18 to 24 hours. During convalescence the reaction is not demonstrable. In

¹ Kindly furnished by Dr. Wadsworth.

patients in whom the disease is prolonged by complications the capacity of the skin to react persists. In 7 fatal cases the skin failed to react to C polysaccharide.

Parallel studies of the reaction of the patients' serum with C have confirmed and extended the observations of Tillett and Francis on the appearance of the precipitation phenomenon during the acute stages and its disappearance in recovery.

That the cutaneous and serological reactions are not specific for pneumococcus infection is shown by the results in 29 control cases. 8 patients with infectious febrile diseases not of pneumococcus origin gave responses similar to those noted in pneumonia. 2 patients with non-infectious fevers and 18 of 19 normal individuals failed to give either skin or serum reactions.

These observations emphasize the importance of using separate components of the bacterial cell in the interpretation of cutaneous and serological reactions in pneumonia. The parallelism in results of the skin and serum tests in pneumococcus pneumonia with the somatic carbohydrate C, the significance of the reactions in relation to the clinical course and outcome of the disease, and the frequency of occurrence of both reactions in bacterial infections other than those of pneumococcal origin, are discussed.

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STUDIES ON THE SOMATIC C POLYSACCHARIDE OF PNEUMOCOCCUS

II. THE PRECIPITATION REACTION IN ANIMALS WITH EXPERIMENTALLY INDUCED PNEUMOCOCCIC INFECTION

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It has previously been shown (1, 2) that the serum of individuals acutely ill with lobar pneumonia or certain other febrile diseases, possesses the capacity of precipitating in the presence of the somatic C polysaccharide of *Pneumococcus*. The precipitating action of the serum is demonstrable within 24 hours after onset of pneumonia, persists throughout the active phase of the disease, and disappears within 1 to 3 days following the beginning of recovery. The phenomenon is not a specific response to pneumococcic infection, since it can be demonstrated with the sera of patients suffering from other bacterial diseases. The chemical or immunological nature of this reaction and the relationship of the factors involved to the mechanism of recovery in pneumonia, at present are unknown. It would appear, however, from the non-specific character of the precipitation reaction, that the phenomenon represents in human beings a broad response to infection by a variety of bacterial agents.

With the aim of obtaining more precise knowledge of the nature of the precipitation reaction as found in man, it seemed desirable to determine whether in certain animals a similar phenomenon occurs in response to experimental infection. For this purpose two animal species, rabbits and monkeys, were selected for study. Infection with pneumococci seemed to be the most desirable experimental approach, inasmuch as the phenomenon in man occurs uniformly in pneumococcus pneumonia, and also because experimental pneumococcus infection in both these species may readily be induced.

Goodner (3) has shown that the intradermal inoculation of rabbits

with Type I pneumococci gives rise to a local inflammatory lesion. A symptom complex is produced which is analogous in many of its features to pneumococcus lobar pneumonia in man. A certain number of animals so infected recover after a definite febrile course. Francis and Terrell (4) have demonstrated that in monkeys of the *Macacus cynomolgus* species it is possible to produce experimental pneumonia by intratracheal or intrabronchial inoculation of Type III Pneumococcus. The clinical aspects of experimentally induced infection in monkeys closely resemble those of spontaneous disease in man.

Pneumococcus infections of rabbits and monkeys appeared, therefore, to offer an experimental approach to the study of the C precipitation reaction of serum. The present paper records the occurrence of the phenomenon in these species following infection by pneumococci.

Materials and Methods

Infection of Rabbits.—The technique developed by Goodner (3) was used in these experiments. Normal rabbits weighing from 1500 to 2000 gm. were infected by intracutaneous inoculation of a mouse virulent strain of Pneumococcus. The site of inoculation was the lateral abdominal wall. 4 animals were infected with a Type I; 9 animals with Type VIII; and 11 animals with a rabbit virulent strain of Type III Pneumococcus (SVIII). The amount of inoculum varied between 0.1 and 0.5 cc. of a 6 to 18 hour culture, but was usually 0.2 cc. All of the animals developed large hemorrhagic, edematous lesions at the site of inoculation, which were observed daily until death or recovery of the animal took place. Throughout the period of active infection the recovered animals showed sustained rises in temperature. A variable temperature response was noted in the animals which died. Blood obtained from the marginal ear vein before infection, at frequent intervals during the febrile period, and after recovery, was used for serological tests.

In order to test the effect of reinfection on the C precipitation reaction of serum, 3 of the recovered animals were given a second intradermal inoculation with a strain different from that used in the initial experiments.

Infection of Monkeys.—The technique elaborated by Francis and Terrell (4) was used for the production of Type III pneumococcus pneumonia in a small series of monkeys. Healthy *Macacus cynomolgus* monkeys were used. The weights of these animals varied between 900 and 5500 gm. 1 to 2 hours after a preliminary intramuscular injection of morphine sulfate, the animals were placed upon a fluoroscopic table. A No. 5 or No. 6 radiopaque catheter was inserted into the trachea and, under the fluoroscopic screen, introduced into a secondary bronchus of a pulmonary lobe. A syringe, containing the sediment from 1 cc. of an 18 hour blood broth culture of Type III organisms (SVIII strain) suspended in 1 cc. of

sterile corn starch solution, was attached to the catheter and the mixture slowly injected. The catheter was quickly removed, the animal tilted upright for a few minutes, and then returned to its cage. Injections were usually made into the right upper or right middle lobe.

A roentgenogram of the chest of each animal was taken before infection, and daily during the course of the acute disease. A daily record of the rectal temperature was made. Once each day during the acute disease and frequently in convalescence specimens of blood were withdrawn from a leg vein or by cardiac puncture. A small amount of blood was used for culture and white blood count. The remainder of the blood was allowed to clot and the serum, collected by centrifugation, was used in precipitation tests with C.

Owing to a limited supply of this species of monkey, experiments involving infection by routes other than the intrabronchial one were not performed, except in one or two instances. One animal was given an intradermal inoculation of 0.5 cc. of an 18 hour blood broth culture of Type III pneumococci. The progress of the infection was observed and daily bleedings taken until the time of death. A second animal, having recovered 6 months previously from Type III pneumonia, was given an intraperitoneal inoculation of 1 cc. of a culture of the same type. A specimen of blood, obtained 24 hours after infection, was used for precipitation test.

Precipitation Tests.—Sera obtained during the course of infection, as well as in convalescence, were tested for their capacity to precipitate the somatic C polysaccharide. Most of the tests were done with preparations of C of the highest degree of purity obtainable. A few tests were done with more crude lots of C prepared according to the original technique of Tillett and Francis (1). The usual precipitation technique was adopted; 0.2 cc. of serum was mixed with 0.3 cc. of salt and varying dilutions of the C substance added. Occasionally, 0.25 cc. of serum added to an equal volume of C dilution was used. In each case readings were made after 2 hours' incubation at 37°C. and refrigeration overnight.

Results in Rabbits Following Intradermal Pneumococcus Infection

The type of lesion which occurred in rabbits following the intradermal inoculation of Type I pneumococci did not differ materially from that found with Type III or Type VIII infections. A slight erythema was usually observed within 3 to 4 hours at the site of inoculation, which rapidly increased in size and intensity so that within 5 to 7 hours a well marked lesion was present. By 24 hours a large hemorrhagic area, often with purpuric center, and marked edema of the ventral abdominal wall, were noted. Fever was almost invariably present in the animals within 24 hours of infection. In the recovered cases this persisted for 3 to 4 days, whereupon a drop in temperature

occurred, oftentimes sharp in character. Regression of the lesion took place slowly over a variable period of time. 16 of the 24 rabbits succumbed to the infection. All 4 of the Type I-infected animals, 4 of the 9 receiving Type VIII infection, and 8 of 11 rabbits infected with Type III pneumococci died after a period of time varying between 1 and 10 days.

Frequent observations were made upon the capacity of the serum of the infected rabbits to precipitate the pneumococcus C polysaccharide. The serum was tested during the height of the disease and during the period of convalescence of the animals which recovered. In a series of 20 rabbits infected intradermally with Types I, III, or VIII pneumococci, it was found that at no time did the serum of any of the animals give a precipitate when C substance was added in varying concentrations.

In Table I are shown the results obtained in a representative group of 6 rabbits during the period of active infection. 4 of these animals were infected with Type I and the other 2 with Type VIII pneumococci. All of the animals developed large spreading hemorrhagic lesions together with fever. The severity of infection is attested by the fact that only one animal recovered. Control bleedings before inoculation showed that the sera failed to react with C in dilution of 1:4000 to 1:128,000. Subsequent bleedings taken 24, 48, and in one case 72 hours after inoculation also gave negative precipitation tests during the period of active infection. The remaining 14 animals in this group were observed for longer periods of time after infection by either Type III or Type VIII pneumococci. Owing to the severity of the lesion many of the animals soon died, and consequently all could not be observed for a uniform length of time. However, 13 rabbits were alive 2 days after inoculation when bled, 6 rabbits survived 4 days, 2 rabbits lived 8 days, and 1 animal was alive 9 days after infection when a sample of serum was taken. The results of precipitation tests on the various sera, irrespective of the duration or severity of the infection as measured by the survival time, were in all instances completely negative.

In the foregoing experiments on 20 rabbits, 0.2 cc. of culture was given to each animal, and the earliest serological observations were made 24 hours after infection. Under these conditions it is possible that the dose of infecting organism was

either too great or not sufficiently large for the demonstration of the C reaction. Furthermore, it is theoretically possible that during the early development of the lesion the serum is capable of precipitating with the C substance, but rapidly loses this property as the disease progresses. In order to obtain evidence on these

TABLE I

Precipitation of C by Sera of Rabbits Given Intradermal Pneumococcus Infection

Rabbit No.	Dosage of infecting organism	Date	Period after infection	Clinical data	Precipitation of C 1:4000 to 1:128,000
		1935	days		
1-2	Type I Pneumococcus (0.2 cc.)	Feb. 2	—	Control bleeding. Infected	None
		" 3	1	Large lesion. Fever. Bleeding. Death in 24 hrs.	"
1-3	Type I Pneumococcus (0.2 cc.)	" 2	—	Control bleeding. Infected	"
		" 3	1	Large lesion. Fever. Bleeding. Death in 24 hrs.	"
8-87	Type I Pneumococcus (0.2 cc.)	" 2	—	Control bleeding. Infected	"
		" 3	1	Marked lesion. Fever. Bleeding	"
		" 4	2	Moribund. Bleeding. Death in 60 hrs.	"
8-88	Type I Pneumococcus (0.2 cc.)	" 2	—	Control bleeding. Infected	"
		" 3	1	Marked lesion. Fever. Bleeding	"
		" 4	2	" " " "	"
		" 5	3	" " " "	"
				Death in 72 hrs.	
9-7	Type VIII Pneumococcus (0.2 cc.)	Mar. 14	—	Control bleeding. Infected	"
		" 15	1	Marked lesion. Bleeding	"
		" 16	2	" " " " Death in 72 hrs.	"
1-06	Type VIII Pneumococcus (0.2 cc.)	" 14	—	Control bleeding. Infected	"
		" 15	1	Marked lesion. Fever. Bleeding	"
		" 16	2	" " " "	"
				No further observations made	

questions, each of an additional group of 4 rabbits was infected with different amounts of Type VIII culture, and samples of serum were taken at intervals during the development of the lesions. At the end of 4 hours slight lesions were detected, which in 5 hours averaged 3 x 5 cm. Fever was present in all of the

animals after 6 hours. It will be seen in Table II that the sera taken 4 and 6 hours after infection did not precipitate C. Moreover, samples of sera obtained 1, 3, and 11 days after infection, despite the variable dosage, failed to give any detectable reaction.

TABLE II

Precipitation of C by Sera of Rabbits Given Type VIII Intradermal Infection

Rabbit No.	Dosage of infecting organism	Date	Period after infection	Clinical data	Precipitation of C (crude) 1:10 to 1:1000
1935					
1-29	Type VIII Pneumococcus (0.1 cc.)	Apr. 6	—	Control bleeding. Infected	None
		" 6	4 hrs.	Slight lesion. Afebrile. Bleeding	"
		" 6	6 "	Marked lesion. Fever. Bleeding	"
		" 7	24 "	" " " "	"
		" 9	72 "	" " " "	"
				Death in 96 hrs.	
1-30	Type VIII Pneumococcus (0.2 cc.)	" 6	—	Control bleeding. Infected	"
		" 6	4 hrs.	Slight lesion. Afebrile. Bleeding	"
		" 6	6 "	Marked lesion. Fever. Bleeding	"
		" 7	24 "	" " " "	"
		" 9	72 "	Lesion healing. " "	"
				" " Afebrile. "	"
1-31	Type VIII Pneumococcus (0.3 cc.)	" 6	—	Control bleeding. Infected	"
		" 6	4 hrs.	Slight lesion. Afebrile. Bleeding	"
		" 6	6 "	Marked lesion. Bleeding	"
		" 7	24 "	" " Fever. Bleeding	"
		" 9	72 "	Lesion healing. " "	"
				" " Afebrile. "	"
1-32	Type VIII Pneumococcus (0.5 cc.)	" 6	—	Control bleeding. Infected	"
		" 6	4 hrs.	Slight lesion. Fever. Bleeding	"
		" 6	6 "	Marked lesion. Fever. Bleeding	"
		" 7	24 "	" " " "	"
		" 9	72 "	Lesion healing. " "	"
				" " " "	"

Tillett and Francis, in their original observations, suggested that a possible interpretation of the C reaction in man might be the so called anamnestic reaction of Cole (5). Applying these principles, they postulated that the C precipitation phenomenon might be conditioned

by a previous exposure of the individual to the same or closely allied bacterial antigen. In the light of these suggestions it seemed of interest to test the effect of a second inoculation in a group of rabbits recovered from an intradermal pneumococcus infection. Accordingly, 3 rabbits which had survived a Type VIII pneumococcus infection given 17 days previously, were reinoculated with 0.2 cc. of Type III culture. Typical lesions were produced and only one animal survived 8 days. Observations made on 3 animals 2 days after infection, on 2 animals 4 days after inoculation, and on the animal which survived 8 days showed that in no instance did the serum precipitate on the addition of the C polysaccharide. Thus, under the conditions of these experiments it was not possible to demonstrate that previous exposure had any effect on the negative precipitation reactions of rabbits reinfected with *Pneumococcus*.

Results of Precipitation Tests in Monkeys Following Pneumococcus Infection

In 4 monkeys of the *Macacus cynomolgus* species experimental pneumonia was produced by the intrabronchial inoculation of a culture of Type III pneumococcus. The course of the infection was identical to that found in a larger series of monkeys by Francis and Terrell, who suggested that the clinical aspects of the experimental disease closely resembled pneumococcus lobar pneumonia in man. Tests made with the serum of these animals have shown that a positive precipitation reaction is demonstrable soon after infection, that the precipitating action of the serum persists for 2 to 3 days, and disappears abruptly if recovery of the animal occurs. The results of precipitation tests with the serum of all the animals were positive throughout the period of active infection. Moreover, the phenomenon was present early in the disease, *i.e.*, within the first 24 hours after inoculation. While positive blood cultures were demonstrated in 3 of the animals during the period in which the precipitation test was positive, no apparent relationship between septicemia and the serum reaction could be discovered. One animal, No. 1-46, did not develop a positive blood culture during the entire infection, yet the serum precipitated C strongly. In animal 1-37 a bacteremia was found to persist after the C reaction had disappeared.

Two animals succumbed to the infection. The precipitating reaction of the sera of both of these animals was demonstrable up to the time of death. The remaining 2 animals recovered after a short period of illness without complications. During the recovery period the precipitation tests were negative.

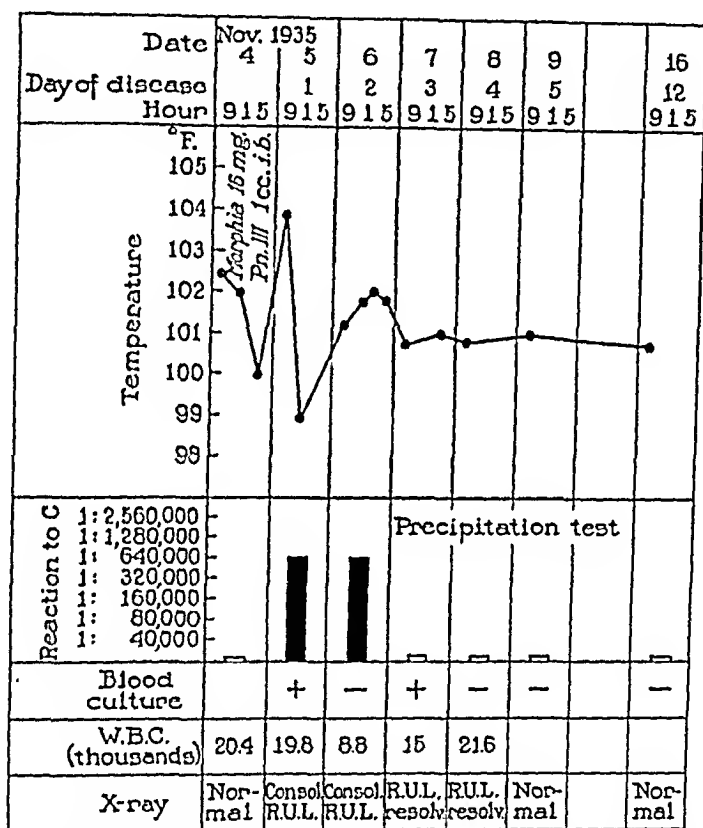


CHART 1. *M. cynomolgus* 1-37. Weight 1250 gm. Type III pneumococcus pneumonia.

The following protocols illustrate the experimental conditions and results obtained in Type III pneumococcus pneumonia.

Monkey 1-37, after preliminary x-ray and intramuscular injection of 10 mg. of morphine sulfate, was inoculated with 1 cc. of Type III pneumococcus culture suspended in 1 cc. of sterile corn starch. The following day, 20 hours after inocu-

lation, a sample of blood was obtained from the femoral vein for culture, blood count, and precipitation tests. X-ray showed marked consolidation of the right upper lobe. The blood culture, 1 drop of blood in 5 cc. of beef infusion broth, pH 7.8, was positive for *Pneumococcus* Type III. The serum on this date precipitated C in dilution of 1:640,000. Each day thereafter these procedures were repeated. On the 2nd day of disease the lung lesion showed signs of resolution by x-ray, the

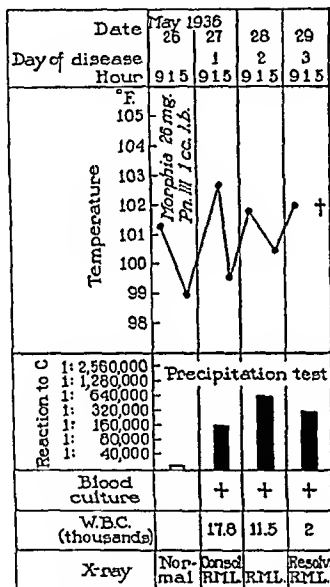


CHART 2. *M. cynomolgus* 1-44. Weight 5540 gm. Type III pneumococcus pneumonia.

blood culture was negative, and the serum precipitated with C in equally high titer. On the 3rd day resolution was well advanced, but although the blood culture was found to be positive, no C reaction could be demonstrated. On the 4th, 5th, and 12th days after inoculation the serum test remained negative. These findings are graphically represented in Chart 1.

Monkey 1-44 was given 26 mg. of morphine sulfate preparatory to the intra-

bronchial inoculation of Type III culture. 23 hours later consolidation of the right middle lobe was found by x-ray. A specimen of blood obtained by cardiac puncture showed approximately 40 colonies of Type III *Pneumococcus* per cc. The serum was found to precipitate C in dilution of 1:160,000. The following day the animal was very ill. Consolidation was confined to the right lung, blood culture remained positive, and the serum now precipitated with C when the latter

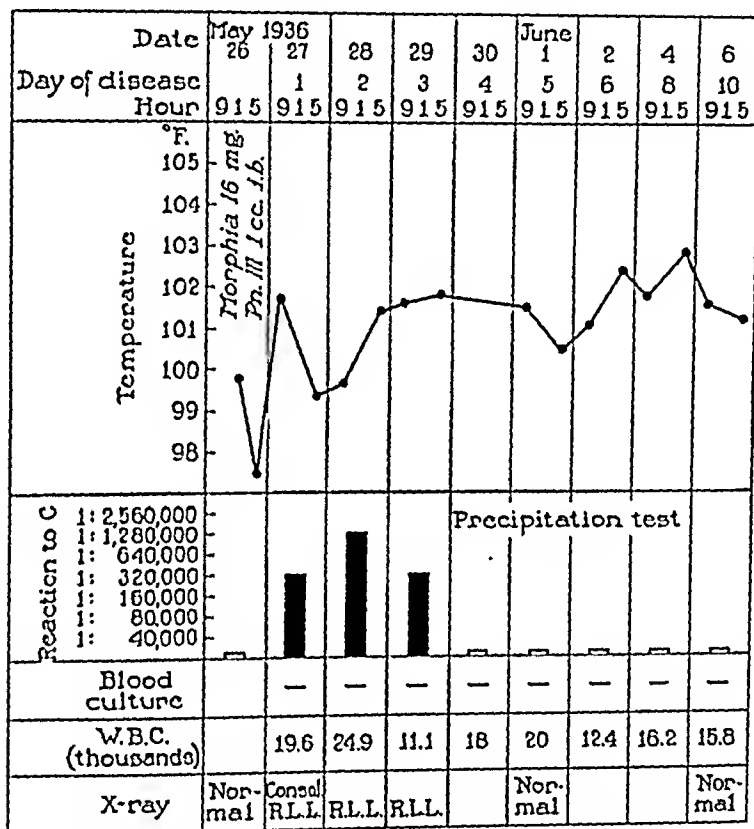


CHART 3. *M. cynomolgus* 1-46. Weight 3900 gm. Type III pneumococcus pneumonia.

was added in dilutions as high as 1:640,000. On the 3rd day of disease the animal's condition was much worse although the lung lesion had begun to resolve. A sharp drop in the total white blood count was observed. The precipitation test remained positive. The animal died following cardiac puncture. Autopsy showed involvement of the right middle lobe with evidences of resolution and, in addition, a traumatic hemopericardium. Chart 2 depicts the course of disease in this monkey.

Extension of the pneumonia occurred to the right lower lobe. Two samples of serum taken 20 and 48 hours after inoculation precipitated the C substance in titers of 1:640,000. The animal died on the 3rd day. Autopsy showed extensive involvement of the right upper and lower lobes.

Two monkeys were given pneumococcus infections by routes other than the intrabronchial one, in order to determine whether pulmonary consolidation was an essential conditioning factor for the demonstration of the C reaction. One monkey received an intradermal and the other an intraperitoneal inoculation of Type III pneumococci. The following protocols describe the results obtained.

Monkey 1-38 was prepared by the intramuscular injection of 16 mg. of morphine sulfate. 2 hours later it was given 0.5 cc. of Type III culture into a shaved portion of the lateral abdominal wall. The following day only localized induration was noted at the site of inoculation, but bacteremia was present. A sample of serum was found to precipitate C in dilution of 1:640,000. On the 2nd day the titer had dropped to 1:160,000. By the 3rd day the serological reaction had disappeared. The animal died on the 4th day. A large subcutaneous abscess yielding a pure culture of Type III pneumococcus was found at autopsy. The lungs were normal in the gross. Chart 4 depicts the findings in this experiment.

After a preliminary injection of 16 mg. morphia, monkey 1-45 (No. 1-37 in previous experiment) was given 1 cc. of a 6 hour culture of Type III pneumococcus into the peritoneal cavity. The following morning the animal was listless, would not eat, and was obviously sick. A specimen of blood taken from the heart gave the precipitation reaction in dilution of 1:160,000 of the C. Blood culture at this time was negative. The animal died shortly after the bleeding. Autopsy showed the lungs to be normal on gross examination. The abdominal cavity revealed an extensive peritonitis. Pneumococci were seen in stained films of the peritoneal fluid, and Type III organisms were recovered in pure culture.

DISCUSSION

The results reported in the present communication demonstrate that the sera of rabbits infected intradermally with pneumococci, and the sera of monkeys given an experimental pneumonia, differ sharply in their capacity to precipitate pneumococcus fraction C. In rabbits inoculated with either Type I, III, or VIII pneumococci a severe febrile infection is produced which frequently results in death of the animal after 2 to 3 days. Attention has been called by Goodner to the analogies between this so called dermal pneumonia and pneumococcus lobar pneumonia in man. However, the sera of infected rabbits fail

to give the C precipitation reaction which is found uniformly in the sera of pneumonia patients during the acute disease. Efforts to demonstrate the phenomenon in the sera of rabbits early in the disease, as well as in convalescence, were unsuccessful. In addition, it was found that a previous pneumococcus infection apparently did not alter an animal's response to a second intradermal infection as measured by the precipitation reaction.

In contrast to the results obtained in rabbits the findings in monkeys corresponded closely with those found in human lobar pneumonia. Within the first 24 hours following the intrabronchial inoculation of Type III pneumococci the sera of the infected animals precipitated with the C substance in high dilutions. The precipitating action of the serum persisted for 2 to 3 days during the period of active infection. Although variations in the precipitation titer were frequently noted, the sera nearly always precipitated C when the latter substance was added in dilution of 1:320,000, less often in dilution of 1:640,000, and in one observation as high as 1:1,280,000. With recovery of the animal the reactivity of the serum with C disappeared almost as abruptly as it had appeared with the onset of disease. After the 2nd or 3rd day of disease the phenomenon could not be demonstrated. Although no tests were available for determining the onset of recovery, the disappearance of the C reaction seemed to coincide roughly with the beginning of resolution as found by x-ray. The height of the febrile response, or the presence of demonstrable bacteremia, apparently had little influence on the capacity of the serum to react with the C substance in precipitation tests.

It is evident from the results of experiments on monkeys, one of which received an intradermal and the other an intraperitoneal inoculation of pneumococci, that the C precipitation phenomenon can be demonstrated in infections of tissues other than the lungs. Whether or not a similar response occurs in monkeys with other bacterial infections is at present unknown, but this seems not improbable in view of the occurrence of the phenomenon in a number of different types of human infection.

The foregoing studies demonstrate the variable responses of two different hosts to the same bacterial agent. In one species, the monkey, pneumococcus infection was accompanied by the demonstra-

tion of certain changes in the serum during the acute period of illness. In the other, the rabbit, these changes were not observed. These observations suggest that during infection in the monkey either some newly formed substance or some alteration occurs in the serum which renders it reactive in precipitation tests with this particular polysaccharide derived from *Pneumococcus*. Assuming this to be the case, then the failure to demonstrate the phenomenon in the rabbit might be explained either by the absence of or qualitative differences in the changes in the serum during infection. Experiments bearing upon these and other questions concerning the mechanism of the serum reaction with C are at present in progress and will be reported in a subsequent paper.

SUMMARY AND CONCLUSIONS

The capacity of the serum of rabbits following intradermal pneumococcus infections to precipitate in the presence of pneumococcus C polysaccharide has been studied during the resultant periods of active infection and during recovery. In rabbits infected with Type I, III, or VIII pneumococci, large hemorrhagic lesions are produced which frequently bring about death of the animals after a febrile illness of 3 to 4 days. Repeated precipitation tests with the sera of these animals have been uniformly and consistently negative, not only during the acute illness but in the recovery period as well.

On the other hand, the sera of monkeys of the *Macacus cynomolgus* species actively ill with experimental Type III pneumonia have been shown to react in precipitation tests with the C substance. The serum reaction appears within the first 24 hours after infection, remains positive in high titer for 2 to 3 days during the acute illness, and disappears with the onset of recovery. The precipitation reaction with C also occurs with the sera of monkeys following intradermal and intraperitoneal infection with pneumococci. The results of precipitation tests of the serum of monkeys during experimental pneumonia are similar to those obtained with the sera of patients suffering from pneumococcus lobar pneumonia.

From the results of these studies it would appear improbable that the demonstration of the serum precipitation phenomenon with C polysaccharide in monkeys, and possibly also in man, is conditioned by previous exposure to pneumococcus antigen.

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EXPERIMENTAL ATTEMPTS TO INCREASE THE BLOOD SUPPLY TO THE DOG'S HEART BY MEANS OF CORONARY SINUS OCCLUSION*

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PLATES 4 AND 5

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This report deals with experimental attempts to alter the blood supply of the dog's heart in such manner as to render it less susceptible to the infarctions which follow sudden occlusion of the anterior descending branch of the left coronary artery.¹ Inasmuch as the ultimate objective of this work is its application to those conditions in the human heart which are associated with myocardial ischemia (angina pectoris of vascular origin generally due to sclerotic or thrombotic coronary occlusion), three practical desiderata were kept in mind: (a) the production of an adequate functional increase in the blood supply to the heart; (b) the employment of a method requiring a minimum of manipulation; (c) the attainment of the desired results after a minimum lapse of time.

In 1921, Gross (1) showed that there are three vascular mechanisms in the blood supply to the human heart which probably serve as compensatory means to ward off the results following coronary artery narrowing or occlusion. The first and most important of these mechanisms is a gradual and consistent widening of intramyocardial anastomotic channels which occurs with increasing age. A conspicuous portion of these anastomotic channels are situated in the interventricular septum. The second compensatory mechanism is the age period development of rami telae adiposae, vessels which lie in the epicardial mantle and anastomose with the myocardial vascular ramifications on the one hand, and with periaortic

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¹ This vessel will be referred to in this report as the left anterior descending branch.

and peripulmonic vessels on the other. The third mechanism is the existence of anastomoses between the myocardial and pericardial vascular ramifications and extracardiac vessels (bronchial arteries, arteriae mammae internae, and diaphragmatic vessels). These studies were subsequently confirmed and extended by Campbell (2), Davis (3), Robertson (4), Hudson, Moritz, and Wearn (5), and by Gross and Kugel (6). That these mechanisms may at times adequately compensate for coronary artery occlusion was suggested by Gross (1) in a series of injection studies on the human heart. Apparently, the adequacy of the compensatory mechanisms depends upon the slowness of the occluding process in the coronary arteries or their branches.

A fourth possible compensatory mechanism which has been emphasized by Wearn (7) is the so called Thebesian backflow. This author believes that under certain circumstances the heart may be nourished by means of retrograde circulation by way of the Thebesian vessels. This theory has received considerable attention and has been both supported and refuted by many experiments.

Recently, augmentation of these extracardiac anastomoses by means of epicardiectomy (stripping of the epicardium) was attempted by Beck, Tichy, and Moritz (8), and by Beck and Tichy (9). Simultaneously, and in graded stages, a gradual occlusion of the coronary arteries and their branches was produced. Some of the dogs tolerated this procedure to a point of considerable narrowing of the coronary bed. No statistical data of the mortality rate are presented, although the authors indicate that this was high. Encouraged by these observations, Beck (10) engrafted the left pectoral muscle on the heart of a patient in whom a diagnosis of coronary sclerosis and angina pectoris was made. 3½ months after the operation the patient was alive and claimed to be well.

More recently, Robertson (11) carried out experiments in dogs in an effort to determine the validity of the Thebesian backflow theory. The coronary sinus, main veins, and coronary arteries were tied off in several stages. In a typical experiment the procedure lasted approximately 6 months. Robertson observed that the venous ligatures were well tolerated by all animals. Following the first coronary artery ligation which closed the main trunks at their origin, the dogs survived for at least some hours. The majority, however, died within 24 to 48 hours. Three showed no ill effects (no data are furnished on the number of animals used). From his experiments and microscopic studies, Robertson concluded that little or no nutritive function developed in the Thebesian vessels but that this depends on the pericardial adhesions.

None of the above mentioned authors reports control experiments on gradual occlusion of the coronary bed without previously or simultaneously producing pericardial adhesions. Inasmuch as gradual coronary artery occlusion by itself leads to compensatory dilatation of intramyocardial anastomoses, the interpretation of their results is open to question. In a recent discussion on the factors concerned

in the development of collateral coronary circulation during slow coronary occlusion, Wiggers (12) has stressed the importance of pressure gradients in distending minute potential communications. Furthermore, Beck and Tichy (9) have already found that vascularization of the myocardium from a collateral bed was slight, and in some experiments, completely absent if the coronary circulation was normal. It seems, therefore, that before applying the epicardiectomy or cardiopexy procedures to the human heart it would be advisable to obtain direct experimental evidence that such pericardial adhesions alone (*i.e.*, without simultaneous coronary artery occlusion) lead to sufficient augmentation of the cardiac vasculature to permit of subsequent sudden coronary artery occlusion with a decrease in the extent of infarction. Apart from these objections, however, the authors themselves observe that the procedures are extremely hazardous and time-consuming. They, therefore, fail to fulfill the criteria considered necessary for a practical method which could be applied to the human heart.

The well known clinical observation that anginal pains not infrequently disappear during an attack of right sided heart failure led one of us to the thought that increased tension in the coronary sinus might accomplish the same result. Because of this and other theoretical considerations as well as anatomical observations, it appeared desirable to determine whether an increase in intramyocardial collateral circulation would follow coronary sinus obturation, and if so, whether the increase was associated with functional enhancement of the myocardial nutrition. In previous communications we have briefly described the results obtained in dogs by the employment of this method (13), the details of a relatively simple technique for occluding the coronary sinus (14), and the electrocardiographic (15) changes which follow complete and partial occlusion of the coronary sinus by various techniques. We have shown that the procedures employed by us can be performed within 20 minutes, and apparently produce a rapid increase in the extent of the coronary bed in the dog's heart. Furthermore, the dilatation of the intramyocardial collateral circulatory channels thus produced is apparently so extensive and abundant that in the majority of otherwise normal dog hearts prepared in this manner it becomes difficult or impossible to induce in-

farcion by subsequent sudden occlusion (division between ligatures) of the left anterior descending branch 2 cm. below the aortic ostium of the left circumflex coronary artery. It is the purpose of this report to describe in greater detail the results obtained in a larger series of animals and to add a number of observations on the effects of the various procedures employed to produce complete or partial occlusion of the coronary sinus, and on their effectiveness in preventing or minimizing the results of subsequent acute occlusions of the left anterior descending branch.

Methods

The methods employed in these experiments are listed in Table I. The dogs weighed approximately 15 kilos. All procedures were carried out under pernoston or nembutal anesthesia. The method of approaching the coronary sinus has been described in detail in a previous report (14). With the dog under anesthesia, a skin incision is made in the right fifth intercostal space extending from the border of the sternum to the mid-axillary line. The chest cavity is entered through this incision and artificial respiration is employed. Care must be taken to avoid hyperventilation or insufficient aeration. After deflation of the right lung by compression with moist pads, an incision 6 cm. long is made in the pericardium parallel to and 0.5 cm. anterior to the phrenic nerve. The incision should extend 1 cm. cephalad to the atrioventricular sulcus. This exposes the coronary sinus. Ligature is effected by passing a blunt needle deep to the coronary sinus in an oblique direction. The point of entry of the needle should be located on the atrial side about 1 cm. from a dimple or puckering which marks the ostium of the coronary sinus. The point of emergence of the needle should lie on the ventricular side 0.5 cm. from the dimple.

As may be seen from Table I, escharotics were used in some experiments in order to produce thrombosis of this vessel, or such considerable perivenous sclerosis as would tend to narrow or occlude the vessel from without. 1 to 2 cc. of the escharotic were injected around the mouth of the coronary sinus (perisinus injection) or within its lumen. The escharotics used were 5 per cent and 10 per cent aqueous solutions of sodium morrhuate, 30 per cent aqueous solution of sodium salicylate, and tincture of green soap (U. S. P.). In order to introduce the escharotic into the coronary sinus, several methods were employed. One was the introduction of a cannula transauricularly. The cannula is fashioned with a pear-shaped extremity in order to prevent the escharotic from being washed out of the coronary sinus by blood flow. The cannula, filled with the escharotic, is introduced through an opening in the right atrium around which there has first been placed a purse-string suture. With little experience the cannula is inserted into the mouth of the coronary sinus, at which site it may be visualized and felt manually (through the visceral pericardium). The cannula is kept in place and 1 to 2 cc. of the escharotic

is injected into the coronary sinus. After 10 minutes, the cannula is removed and the purse-string suture in the atrium closed off.

TABLE I

Operative Mortality Rate Associated with Coronary Sinus Occlusion in Dogs

	No. of dogs	No. surviving	No. dying within 24 hrs.	Operative mortality rate (within 24 hrs.) per cent
Complete coronary sinus occlusion				
Ligation	46	31	15	33
Dissection and ligation	16	9	7	44
Ligation and injection*	8	4	4	50
Intracoronary injection* through transaortic cannula	2	1	1	50
Intracoronary injection* with temporary occlusion†	17	7	10	59
Total.....	89	52	37	= 42
Partial coronary sinus occlusion				
Ligation	6	6	0	0
Dissection and ligation	2	2	0	0
Ligation and injection*	4	3	1	25
Pericoronary injection*	14	12	2	14
Intracoronary injection* through transaortic cannula	2	2	0	0
Intracoronary injection* with temporary occlusion	11	10	1	9
Total.....	39	35	4	= 10
Unsuccessful coronary sinus occlusion				
Ligation	7	7	0	0
Dissection and ligation	1	1	0	0
Ligation and injection*	3	3	0	0
Pericoronary injection*	1	0	1	100
Intracoronary injection* through transaortic cannula	4	2	2	50
Intracoronary injection* with temporary occlusion	5	2	3	60
Total.....	21	15	6	= 29

* Escharotics were injected as indicated in text.

† Temporary occlusion by ligature or digital.

Another method of temporarily retaining the escharotic in the coronary sinus was by digital compression of the vessel, or better still, by a temporary ligature. In these experiments, the escharotic is introduced into the distal portion of a coronary sinus by means of a hypodermic syringe. After 5 to 10 minutes the occlusion is released. Recently, we have been attempting to inject the escharotic through a cannula introduced through the right jugular vein. Our observations following the use of this procedure are as yet incomplete.

In order to occlude the left anterior descending coronary branch, the left chest is entered through the third intercostal space, artificial respiration being simultaneously applied. The pericardium is exposed from above the conus to the apex for a distance of 4 cm. from the latter. After carefully exposing the left anterior descending branch and its venae comites, approximately 2 cm. from the left circumflex coronary ostium, two suture ligatures are placed about it and the artery is divided between them. It is most important to emphasize the necessity of cutting the artery between ligatures since ligature alone of the artery is not infrequently unsuccessful for the production of complete occlusion.

In some of the experiments, topical injections or applications of alcohol or cocaine solutions were made around the region of the coronary artery occlusion in an effort to avoid possible nervous factors influencing the vascular system. The results of these experiments were inconclusive.

After death of the dogs, autopsy was performed and the hearts were injected under standardized conditions with a barium sulfate suspension in gelatine using the method described by Gross (1, 16). It is to be noted that this method injects the coronary tree as far as the arterioles and precapillaries. Since the injection mass does not reach the capillary bed, this method offers more reliable information concerning the extent of the arterial tree than one which fills the capillary bed and enters the veins, such as used by Beck and his associates. The barium sulfate-injected hearts were fixed in 10 per cent neutralized formalin saline.² Roentgenograms were then taken of the intact heart as well as of serial slices of the specimen 7 mm. thick, cut transversely from base to apex.

Electrocardiographic records were taken before and during anesthesia, when the pericardium was opened, after dissection of the vessel or after passing the ligatures, immediately after tying and cutting the vessels, after the closure of the chest, and finally at various intervals (hourly or daily) until the dog died or was sacrificed. The electrocardiographic changes following coronary sinus occlusion have already been reported elsewhere in detail (Gross, Silverman, and Master (15)). Since these findings are of some significance in elucidating the results obtained in these experiments, reference will be made to them in this report. In addition, observations on the electrocardiographic changes following occlusion of the left anterior descending branch will be mentioned briefly. This will be taken up more fully in a subsequent report.

² Solution of formaldehyde U. S. P. 10 parts, 1 per cent sodium chloride solution 90 parts. This solution is rendered neutral with a weak alkali.

EXPERIMENTAL

1. *Results Following Sudden Complete Occlusion of the Left Anterior Descending Branch 2 Cm. below the Ostium of the Left Circumflex Coronary Artery*

In order to compare the results of sudden complete coronary artery occlusion upon dogs which had withstood coronary sinus obliteration, with control animals in which no previous manipulation had been carried out, sudden occlusion of the left anterior descending coronary branch was produced in 53 dogs. The operation was performed under pernoston or nembutal anesthesia in the manner previously

TABLE II

Results of Sudden Left Anterior Descending Branch Occlusion in Control Dogs and in Dogs Prepared by Preliminary Coronary Sinus Occlusion

Preliminary procedure	No. of dogs	No. dying within 24 hrs.	Operative mortality rate (within 24 hrs.)	No. of dogs surviving sudden L.A.D.* occlusion	Average size of infarct
			per cent		
Control	53	28	53	25	24 dogs, 5 x 5 cm. 1 dog, 1 x 1 cm.
Complete coronary sinus occlusion	29	16	55	13	7 dogs, no infarct 2 dogs, 1 x 1 cm. 4 dogs, 3 x 3 cm.
Partial coronary sinus occlusion	29	9	31	20	2 dogs, no infarct 2 dogs, 1 x 1 cm. 7 dogs, 3 x 3 cm. 9 dogs, 5 x 5 cm.
Unsuccessful coronary sinus occlusion	13	9	67	4	3 dogs, 3 x 3 cm. 1 dog, 5 x 5 cm.

* Left anterior descending branch.

described. 28 of these dogs died within 24 hours of the operation (Table II). Death was generally due either to surgical shock or to ventricular fibrillation. The significance of these two factors will be taken up in the discussion. It was found that when dogs survived for over 24 hours the mortality rate, due to the operative procedure, dropped sharply. In this report, therefore, the mortality rate will refer to death taking place within 24 hours of the operative procedure.

In a series of studies at present being carried on by Gross, Mendlowitz, and Schauer (17), the cardiovascular dynamics in these dogs were carefully investigated. In brief, the outstanding features following such occlusion of the left anterior descending branch are diminished cardiac output and delay in cyanide

circulation time. In a number of hearts there was present a darkening of the anterior wall (beginning infarction). On injection, the vascular tree usually showed a large filling defect. In all cases the remainder of the coronary tree was similar to that found in normal animals.

Twenty-five dogs were sacrificed at periods ranging from 36 hours to 12 weeks. Most of the dogs were permitted to survive 1 week. In 24 of these dogs, the hearts showed a large infarcted area on the surface of the left ventricle bordering the interventricular septum and apex. These infarctions averaged 5 x 5 cm. on the surface. In one heart, the infarct measured 1 x 1 cm. The injection invariably disclosed a filling defect of the coronary tree (Figs. 1, 2, 3, and 4) which corresponded roughly to the size of the infarct. In ten dogs the remaining vasculature was similar to that seen in the intact heart. In seven, the arterial tree was somewhat dilated, suggesting the possibility of a beginning compensatory process.

The electrocardiographic changes which were most frequently associated with the left anterior descending branch occlusion consisted of RT_1 elevations and ST_2 and ST_3 depressions. In approximately one-third of the dogs these electrocardiographic changes occurred in such combinations as were not found in dogs which had previously undergone coronary sinus occlusion.

2. Results Following Sudden Complete Occlusion of the Coronary Sinus

The coronary sinus was completely occluded³ in 89 dogs by the methods listed in Table I. Immediately after occluding the coronary sinus, whether by ligature or cannula, a turgidity was noticed of the entire heart. The superficial veins became engorged and the left ventricle became cyanotic up to and slightly beyond the interventricular grooves. Occasionally, ecchymotic spots appeared on the surface of the left ventricle. The right ventricle retained its normal color except for a strip adjacent to the interventricular grooves, particularly on the posterior aspect of the heart.

Thirty-seven of these dogs died in less than 24 hours. The remaining dogs survived or were permitted to survive for a period up to 6 months after the coronary sinus occlusion. The average survival was approximately 4 weeks. Death under 24 hours was generally associated with evidence of surgical shock, although cardiac asphyxia due to the sudden venous congestion may have played a rôle. In this group of experiments, occlusion by the ligature method was associated with the lowest mortality rate. It must be noted, however, that in this experiment the attempts made in the use of the escharotic method were relatively crude as we were endeavoring to determine the optimum escharotic fluid and the effective dose to be employed. As a result, the escharotic method showed a mortality rate which was probably in excess of that which could be secured by the use of milder escharotics. In a number of dogs the venous thrombosis was so extensive and the necrosis of the myocardium so marked that this undoubtedly led to the death of the animal.

³ The extent of coronary sinus occlusion was always determined at autopsy.

Immediately following complete occlusion of the coronary sinus, the following electrocardiographic changes were consistently observed: elevation of the RT transition, notching and downward direction of the main QRS deflection, inversion of the T wave, and temporary slowing of the heart rate. Large T waves of a transitory nature were occasionally noted. Partial heart block was noted in two dogs. The slowing of the heart rate lasted a few minutes and took place only when ligation was employed. The fact that it did not take place when escharotics alone were used indicates that the slowing is not due to irritation at the mouth of the coronary sinus. When the coronary sinus was dissected before ligation, the immediate records showed no downward direction of the QRS deflection, notching of the QRS was less frequent, the T waves were inverted and the RT transition was elevated. When escharotics alone were used for the occlusion, the electrocardiographic changes were inconstant. All electrocardiographic changes tended to return to normal within 2 to 4 weeks.

After death of the dogs, the hearts were injected with a barium sulfate suspension in gelatine and roentgenograms were taken. In most instances, the coronary artery tree showed considerable dilatation (Fig. 5), as compared to that found in intact dogs' hearts (Fig. 1). The vascular channels were on the whole wider, and more vessels were generally visible.

In order to determine whether this increase in the extent of the coronary tree was associated with a corresponding functional improvement in the nutrition of the myocardium, dogs which had survived coronary sinus occlusion were submitted to subsequent sudden left anterior descending branch occlusion. If it could be shown that the incidence and extent of infarction is thereby reduced, this would suggest that the widening of the vascular channels is associated with a better perfusion during life or that a more extensive shunt mechanism is provided so that additional vascular channels may more easily take over the function of the occluded vessels.

3. Results Following Complete Occlusion of the Left Anterior Descending Branch 2 Cm. below the Ostium of the Left Circumflex Coronary Artery in Dogs Which Had Survived Complete Coronary Sinus Occlusion

In 29 dogs which had survived complete coronary sinus occlusion for a period of time varying from 1 to 8 weeks (average 4 weeks), sudden left anterior descending branch occlusion was performed in the manner described above (Table II). Sixteen of these dogs succumbed during the first 24 hours following the left anterior descending branch occlusion. No infarct or discoloration was seen on the surface of the heart. On injection, most of the hearts showed an enrichment in vasculature. Only one heart showed a filling defect on injection.

Thirteen dogs survived or were permitted to live for a period of time ranging from 3 days to 6 weeks (average 1 week) following the left anterior descending branch occlusion. In seven of these, no infarcts were present (Figs. 5, 6, 7, and 8). In two, a very small infarct measuring 1 x 1 cm. was found. In four, infarcts definitely smaller than those almost invariably found in the control animals, were present in the dogs' hearts. These averaged 3 x 3 cm. on the surface. The only filling defects at times noted on injection were those corresponding to the infarcts. Almost invariably the coronary tree was considerably enriched and dilated.

Unfortunately electrocardiographic studies were made only on those dogs which died in less than 24 hours or in those which presented small infarcts. The most frequent findings were elevation of RT_1 and depression of ST_2 and ST_3 . The incidence of these changes was similar to that found in the control group 1. On the other hand, certain combinations of changes, e.g., elevation of RT_1 , ST_2 , and ST_3 , etc. were not found. This therefore, also suggested an influence of the preliminary coronary sinus occlusion.

In spite of the improvement in the nutrition of the myocardium as indicated by the decreased incidence and diminished extent of the infarctions, complete coronary sinus occlusion did not lower the mortality rate following subsequent left anterior descending branch occlusion (Table II). Undoubtedly, therefore, the factors responsible for death were not inhibited by the enrichment of the coronary tree. What these factors may be, and the relative importance of each will also be taken up in the discussion.

4. Results Following Partial Occlusion of the Coronary Sinus

In 39 dogs partial coronary sinus occlusion was produced by the procedures listed in Table I. Four of these dogs died within 24 hours. Six dogs succumbed to snuffles within 4 days to 6 weeks after the operation. The hearts were injected and showed a somewhat increased vascular bed. This, however, was not as consistent as in those in which the coronary sinus was completely occluded. The remaining dogs were permitted to survive for periods up to 6 weeks (average 1 week) at which time they were submitted to left anterior descending branch occlusion. Electrocardiographic findings in these dogs were qualitatively similar to those described in the dogs in which total coronary sinus occlusion had been produced. The QRS changes, however, were less constant.

5. Results Following Sudden Complete Occlusion of the Left Anterior Descending Branch 2 Cm. below the Ostium of the Left Circumflex Coronary Artery in Dogs Which Survived Partial Coronary Sinus Occlusion

The left anterior descending coronary branch was completely occluded in 29 dogs which survived partial coronary sinus occlusion for periods of from 1 to 6

weeks. In the majority of instances the partial coronary sinus occlusion in this series followed injection of escharotics into or around the coronary sinus. Of the 29 dogs in which sudden occlusion of the left anterior descending branch was produced, nine died within 24 hours after the operation (Table II). In three of these, vascular filling defects, but no discoloration or infarction, was noted.

Of 20 dogs which survived the left anterior descending branch occlusion for periods of from 1 day to 3 weeks (average 1 week) all but two showed an increase in the vascular bed. In two dogs there was no infarction. In two, the infarctions measured 1×1 cm. on the surface, in seven the infarctions averaged approximately 3×3 cm., and in nine the infarction was of the usual extent, averaging approximately 5×5 cm. on the surface. On injection, filling defects were present in the vascular tree corresponding to the presence and size of the infarct. The remainder of the coronary bed was usually increased in extent. The electrocardiographic changes were studied largely in the group of animals showing infarctions. They were similar to those described in group 3.

It appears from the above mentioned findings that in spite of the incomplete coronary sinus occlusion, there occurred a definite though moderate improvement in the nutrition of the heart so that in half of the dogs the infarctions following subsequent sudden left anterior descending branch occlusion were either absent or considerably smaller than those observed in the control group. Moreover, since the mortality rate following the left anterior descending branch occlusion was considerably lower (31 per cent) than in the group in which the preliminary coronary sinus occlusion was complete (55 per cent) or in which the preliminary coronary sinus occlusion was unsuccessful (67 per cent), it would indicate that partial, or perhaps gradual coronary sinus occlusion is the desirable approach to the problem. These observations, however, should be confirmed on a larger series of animals.

6. Results Following Unsuccessful Coronary Sinus Occlusion

The manipulations listed in Table I under the entry Unsuccessful coronary sinus occlusion were carried out in 21 dogs. In some (ligation), the coronary sinus was deliberately manipulated without intention to produce occlusion. The purpose of this was to discover whether dilatation of the coronary tree followed such manipulation, and in order to study the electrocardiographic findings. In the remaining dogs absence of coronary sinus occlusion was due to faulty technique. Electrocardiographic changes in this group were infrequent but were similar qualitatively to those described for the other groups (Nos. 3, 4). Their presence, together with the fact that subsequent left anterior descending branch occlusion appeared to

produce a smaller infarct in some of the dogs, suggested that a transient narrowing of the coronary sinus may have been present following the injection of escharotics. Six of these dogs died in less than 24 hours after operation. Two survived for periods of 1 and 3 weeks, respectively, and were sacrificed for anatomical studies. The remaining dogs were employed for the experiments described in the next group.

7. *Results Following Sudden Complete Occlusion of the Left Anterior Descending Branch 2 Cm. below the Ostium of the Left Circumflex Coronary Artery in Dogs Which Had Survived Unsuccessful Occlusion of the Coronary Sinus*

The left anterior descending branch was completely occluded in thirteen dogs which survived unsuccessful⁴ coronary sinus occlusion for a period of from 1 to 6 weeks. Nine dogs died within 24 hours after the operation (Table II). Vascular defects were present in only two of these. No discoloration of the myocardium or other evidence of infarction was present.

Of four dogs which survived for an average period of 1 week, one showed an infarct measuring 5 x 5 cm. on the surface of the left ventricle and the other three showed smaller infarcts averaging 3 x 3 cm. It is thus seen that even in this group there seems to be a tendency for the infarcts resulting from sudden left anterior descending branch occlusion to be somewhat smaller than those found in the control group 1. Furthermore, as was indicated above, the occasional occurrence of some of the characteristic electrocardiographic changes (confined to those animals in which escharotics had been used), the somewhat more extensive vascular tree, and the lower incidence of vascular filling defects suggest the possibility that a transient occlusion was present in this group. In further support of this view was the perisinus thickening found in several of the hearts in which perisinus infiltration was employed.

DISCUSSION

In appraising the therapeutic value of coronary sinus occlusion as carried out in the experiments herein described, several pertinent facts must be borne in mind. First, the left anterior descending branch occlusion was complete and sudden. These experiments, therefore, differ in this important respect from those reported by Beck and his coworkers and by Robertson. Secondly, the results of such sudden coronary occlusion in unprepared dogs were completely controlled and are well known. In our control series of 53 dogs in which this procedure was carried out, 28 died in less than 24 hours. Roughly speaking, therefore, the mortality rate may be considered to

⁴ As subsequently determined at autopsy.

be approximately 50 per cent. This agrees with the findings of previous investigators. In a number of dogs dying in less than 24 hours there was present evidence of beginning infarction in the heart (myocardial discoloration and early softening). Furthermore, in all the specimens which were injected, a filling defect was noted in the coronary tree. Of the 25 dogs which survived over 24 hours, all showed infarction of the heart and in 24 of these the surface of the infarct averaged 5×5 cm. approximately 1 week following the occlusion. Large filling defects of the coronary tree were noted on injection.

In sharp contrast to these findings are those following left anterior descending branch occlusion in animals which had survived occlusion of the coronary sinus for periods of time varying from 1 to 8 weeks (average 4 weeks). When the coronary sinus occlusion had been complete, irrespective of the technique used for this purpose, there was a pronounced beneficial effect on the incidence and extent of the infarction following complete and sudden left anterior descending branch occlusion. In over half of the dogs no infarction resulted. (Most of the animals were killed approximately 1 week after the left anterior descending branch occlusion.) In the remainder, the infarctions were considerably smaller than expected. The vascularity was on the whole increased and the filling defects on injection were either absent or small.

When the coronary sinus occlusion was incomplete or transient there were still present evidences that the myocardial nutrition had improved. In these animals the infarctions were present almost as frequently as in the control group but in approximately half of them the size of the infarctions as well as the vascular filling defect were considerably diminished. Moreover, in the animals which survived for less than 24 hours, filling defects were generally smaller in size and myocardial discoloration was never noted. One must conclude from these observations that at least in the experimental animal (dog) complete or even partial occlusion of the coronary sinus, whether this be permanent or transient, affords a definite method of anatomically and functionally enriching the coronary bed to such an extent that infarction may be either completely prevented or minimized.

There are several factors which enter into the incidence of mortality in these experiments: surgical trauma (anesthesia, shock, postoperative pneumonia, etc.); injury to the myocardium due to the ischemia following left anterior descending branch occlusion; secondary phenomena of cardiovascular damage (ventricular fibrillation, decreased cardiac output, etc.), and possible injury to the myocardium due to the venous congestion following complete coronary sinus obturation (turgidity of the heart, slowing of heart rate, electrocardiographic changes, etc.). The factors attendant on the surgical procedure itself, *i.e.*, due to anesthesia and thoracotomy, remain more or less constant in all experiments. Opposing the mortality factors relevant to cardiac injury, however, is the enrichment of the circulation following the coronary sinus occlusion. When this coronary sinus occlusion is complete there is evidence that in spite of the functional improvement in myocardial nutrition, the sudden venous congestion itself may be an added trauma. The desideratum, therefore, is a proper balance between the beneficial effects of the improved circulation and the undesirable effects due to possible myocardial injury.

With these considerations in mind, an analysis of the experiments described in this report assumes added significance. As noted above, the mortality rate following sudden complete left anterior descending branch occlusion was 53 per cent. The only group in which this was materially reduced was that in which a preliminary partial coronary sinus occlusion was present (31 per cent). Furthermore, the primary mortality rate of partial coronary sinus occlusion was very low (10 per cent). Of the methods used to produce partial coronary sinus occlusion, perisinus injection of escharotics appears up to the present to be the most satisfactory and to give the most consistent results. If these observations can be confirmed on a larger series of animals it would indicate that a partial occlusion of the coronary sinus strikes a satisfactory balance between the undesirable and beneficial factors. Thus, on the one hand, it is associated with sufficient enrichment of the coronary bed to reduce the mortality rate and the extent of the infarction otherwise following sudden occlusion of the left anterior descending branch and, on the other hand, it either does not damage the myocardium or it does not produce sufficient myocardial damage

to maintain or enhance the mortality rate of the subsequent sudden, complete occlusion of the left anterior descending branch. It is only when the coronary sinus obturation is sudden and complete or when it is inadequate or transient that the mortality rate following sudden occlusion of the left anterior descending branch again rises to the expected level in spite of the fact that there may be some improvement in the vascular nutrition. It appears desirable, therefore, that improved methods be sought for which might produce a partial, or perhaps better, gradual occlusion of the coronary sinus.

In appraising the results of these experiments in terms of application to the human heart, it is to be noted that sudden occlusion of the left anterior descending branch by the methods employed in these experiments is far more drastic than the occurrence of thrombosis in the human heart. The latter occurs in a myocardium which has almost invariably already developed compensatory changes in the coronary tree as a result of gradual coronary narrowing (sclerotic). Furthermore, the thrombotic process itself probably lasts for at least several hours. The beneficial results following the several procedures outlined above, therefore, were obtained under the most adverse of conditions, such as are not encountered in the human heart. Furthermore, it is to be noted that cardiopexy procedures produce little or no vascularization of the myocardium in the intact heart. Assuming, therefore, that some modification of the cardiopexy procedure might lead to beneficial results, this operation should be carried out only when the myocardium is severely damaged. Coronary sinus occlusion, on the other hand, apparently produces a considerable enhancement in the vascular nutrition even of the normal heart. This suggests its possible application both in advanced cases as well as in mild cases of coronary artery disease as a preventative against the results of subsequent arterial occlusion.

SUMMARY AND CONCLUSION

Sudden occlusion of the left anterior descending branch approximately 2 cm. below the ostium of the left circumflex coronary artery in the dog's heart produces a mortality rate of approximately 50 per cent. In dogs weighing approximately 15 kilos surviving more than 24 hours (average 1 week), an infarction is produced which al-

most invariably measures 5 x 5 cm. on surface. Following coronary sinus obturation such secondary sudden occlusion of the left anterior descending branch is followed either by no infarction or by a reduction in the size of the infarct. The success of the procedure, quite apart from the mortality rate, depends upon the completeness of the coronary sinus obturation. On the other hand, sudden and complete coronary sinus obturation by itself is associated with a high operative mortality and apparently does not affect the mortality rate following subsequent sudden left anterior descending branch occlusion. Partial persistent obturation of the coronary sinus, however, is in itself associated with a low operative mortality. Furthermore, its experimental production in dogs appears to lower the mortality rate following subsequent sudden occlusion of the left anterior descending branch and to diminish the extent of the infarction.

In the introduction to this report it was pointed out that there are three important desiderata to the problem of improving the coronary circulation in the human heart. The findings herein reported fulfill these requisites to an encouraging degree. It has been shown that following the outlined procedures, a functional increase in the blood supply to the heart can be produced in a significant proportion of experimental animals, this varying with the nature of the experimental procedure. The manipulation is simple, can be performed in the dog within approximately 20 minutes, and does not lead to appreciable pericardial adhesions. Increase in the nutrition of the myocardium is noted 1 week after the experimental procedure. Although no experiments employing sudden left anterior descending coronary branch occlusion were carried out sooner than 1 week, there is available anatomic evidence that within possibly 24 hours after coronary sinus occlusion a dilatation of the vascular bed occurs. In subsequent experiments attempts will be made to determine whether this early vascular dilatation is adequate to compensate for subsequent sudden left anterior descending branch occlusion.

A discussion is given of the results following various coronary sinus occlusion procedures in which it is indicated that it is desirable to produce a partial or gradual occlusion in order to lower the mortality rate both of the initial procedure as well as of the subsequent sudden arterial occlusion. Experiments thus far reported on cardiopexy

operations are lacking in evidence that they are associated with appreciable improvement in the vascular nutrition of the myocardium.

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EXPLANATION OF PLATES

PLATE 4

FIG. 1. Anteroposterior view of injected dog's heart 9 days after sudden complete occlusion of the left anterior descending branch 2 cm. below the ostium of the left circumflex coronary artery. Arrow marks site of left anterior descending branch occlusion. A, right ventricle; B, interventricular septum; C, left ventricle. Note large vascular filling defect (X) and infarct at apical region.

FIG. 2. Cross section through middle of dog's heart illustrated in Fig. 1. A, right ventricle; B, interventricular septum; C, left ventricle. Note small vascular filling defect (X) and infarct in anterior wall of left ventricle.

FIG. 3. Cross section half way between middle and apex of dog's heart illustrated in Fig. 1. A, right ventricle; B, interventricular septum; C, left ventricle. Note large vascular filling defect (X) and infarct in anterior wall of left ventricle.

FIG. 4. Cross section through apex of dog's heart illustrated in Fig. 1. A, right ventricle; B, interventricular septum; C, left ventricle. Note large vascular filling defect (X) and infarct in anterior wall of left ventricle.

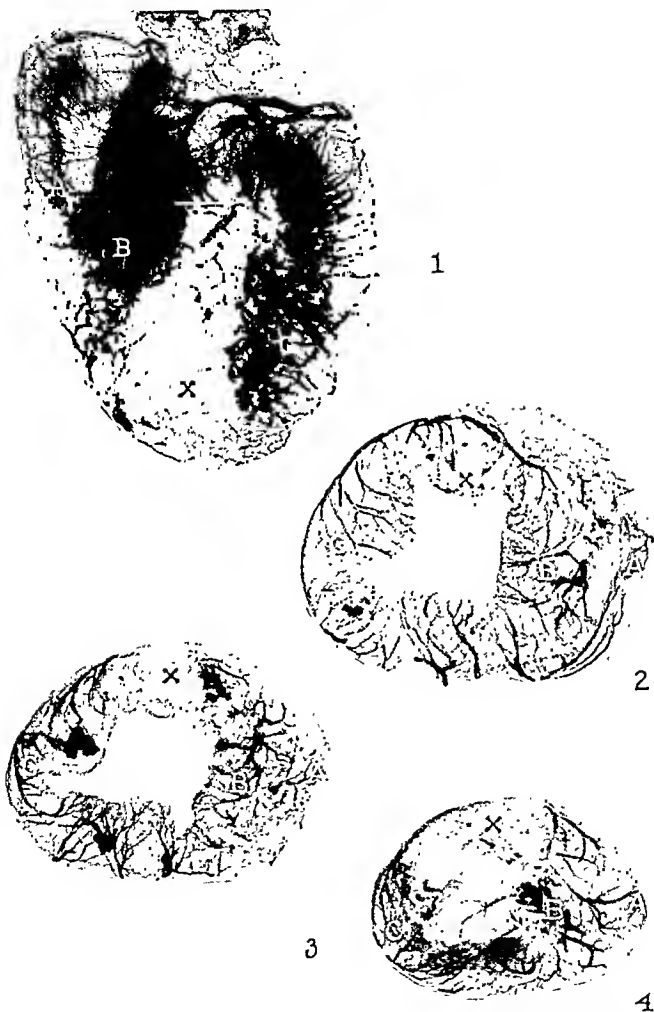
PLATE 5

FIG. 5. Anteroposterior view of injected dog's heart 2 weeks after sudden complete occlusion of the left anterior descending branch 2 cm. below the ostium of the left circumflex coronary artery. 4 weeks prior to the occlusion of the left anterior descending branch, the coronary sinus was completely occluded. Note extensive coronary tree. Arrow marks site of left anterior descending branch occlusion. A, right ventricle; B, interventricular septum; C, left ventricle.

FIG. 6. Cross section through middle of dog's heart illustrated in Fig. 5. Anterior wall is uppermost. A, right ventricle; B, interventricular septum; C, left ventricle. Note absence of filling defect or infarct.

FIG. 7. Cross section half way between middle and apex of dog's heart illustrated in Fig. 5. Anterior wall is uppermost. A, right ventricle; B, interventricular septum; C, left ventricle. Note absence of filling defect or infarct.

FIG. 8. Cross section through apex of dog's heart illustrated in Fig. 5. Anterior wall is uppermost. A, right ventricle; B, interventricular septum; C, left ventricle. Note absence of filling defect or infarct.



(Gross *et al.*: Coronary sinus occlusion)



5



6



7



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IMMUNOLOGICAL AND CHEMICAL INVESTIGATIONS OF VACCINE VIRUS

V. METABOLIC STUDIES OF ELEMENTARY BODIES OF VACCINIA

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The lack of knowledge of the exact nature of viruses has led to a great deal of discussion; certain workers believe that they are inanimate substances, while others look upon them as minute living organisms. According to the first view, a virus is capable of reproduction by a process of autocatalysis; this idea has received considerable support from the work of Stanley (1), who claims to have isolated a crystalline protein having the properties of tobacco mosaic. According to the second conception, viruses are autonomous living agents, which, being incapable of independent existence, are obligate intracellular parasites (2-4). If this conception be correct, it is not unreasonable to suppose that under proper conditions viruses might evidence independent metabolism even though they are capable of multiplication only in the presence of living host cells. With the object of demonstrating such metabolism, several attempts have been made in the past to measure the rate of respiration of viruses.

In attempts to measure the respiration of certain viruses, Bronfenbrenner (5) used a microspirometer, capable of detecting very small amounts of carbon dioxide. He failed to find elahoration of carbon dioxide by hacteriophage. Later, with Reichert (6), he attempted to measure the amount of oxygen consumed and carbon dioxide produced by the viruses of rahies and herpes simplex. Lacking cell-free preparations of these viruses, the authors resorted to the use of emulsions of brain tissue which contained the infectious agents; emulsions of normal brain tissue were employed as controls. They reported that the values obtained for the virus-containing emulsions did not differ significantly from those found for normal tissue. They also found that a certain amount of oxygen was consumed even after the tissues had been stored in the ice box for 6 months, by which time the tissues had lost the property of forming carbon dioxide; the consumption of oxygen was not related to the activity of the virus, continuing even after the virus had been

inactivated with phenol. More recently, Breinl and Glowazky (7) reported that they were able to demonstrate respiration of vaccine virus which had been freed from cells by means of differential centrifugation. Assuming an active metabolism of the virus, it is difficult, however, for one to understand how the figures they give could have been obtained by the methods described. In a brief communication, Kempner (8) has reported that the serum of birds containing the virus of fowl plague absorbs oxygen while the serum from normal fowls does not. This absorption of oxygen is not inhibited by octyl alcohol which quickly stops the absorption of oxygen by red blood cells.

In the past it has been difficult or impossible to obtain appreciable amounts of active virus in a state suitable for metabolic studies. In the case of vaccine virus, however, this difficulty has been overcome, because, by the application of recently developed technics, it is possible to prepare suspensions of elementary bodies of vaccinia which are almost entirely free from other particulate material, to wash the elementary bodies, and to concentrate a suspension of them to any desired degree, (9, 10). Furthermore, although the original preparations contain a few viable bacteria these may be killed with ether without materially harming the virus. Such suspensions of washed elementary bodies which are highly infectious have been used by us for the study of consumption of oxygen and production of acid by vaccine virus.

Methods and Materials

Preparations of Suspensions of Elementary Bodies of Vaccinia.—The manner of preparing suspensions of elementary bodies of vaccinia has already been described in detail (10). The elementary bodies in such suspensions have been subjected to repeated washing in a markedly hypotonic (0.004 M, citric acid-sodium phosphate buffer) solution by means of differential centrifugation, and were free from cells in so far as could be determined by the examination of numerous stained smears. Bacteria and any viable tissue cells that might have remained despite the repeated washings were killed by the addition of an excess of ether to the suspensions which had been reduced to a volume of 15 cc.; the mixture was stored at 4°C. until poured agar plates seeded with 0.1 cc. samples failed to reveal bacterial growth. For use in experiments in which the consumption of oxygen was to be measured the elementary bodies were suspended in 0.066 M phosphate buffer solution of pH 7.2; in experiments in which the production of acid was determined they were suspended in distilled water or Ringer's solution; sodium bicarbonate solution was added at the beginning of the experiment. In either case, before being used they were washed twice with the appropriate solution and finally suspended in 1.4 to 2.0 cc. of it, due precautions being taken to avoid

the introduction of bacteria. For each experiment the elementary bodies obtained from 10 rabbits were used. After the amounts of oxygen consumed and acid produced were estimated, the infectious titers of the preparations were determined by intradermal inoculation in rabbits of serial tenfold dilutions of the suspensions; most of them were active in a dilution of 10^{-9} . At the conclusion of each experiment the elementary bodies were washed 3 times in distilled water, dried *in vacuo* over calcium chloride, and weighed.

Warburg Apparatus.—Oxygen consumed and total acid (carbon dioxide plus fixed acid) produced, as measured by liberation of carbon dioxide from a buffer solution containing sodium bicarbonate and carbon dioxide, were measured in a Warburg apparatus of the usual type (11). Appropriate controls containing all the solutions used were included in each experiment and the figures recorded have been corrected for any pressure change shown by the control.

Tissue Extract Containing a Respiratory Supplement.—As a source of respiratory supplement an extract of normal kidney prepared in the following manner was used. A normal rabbit was killed by the intravenous injection of air, and the kidneys were removed aseptically. These were minced with scissors and ground with alundum and twice their weight of isotonic buffer solution, pH 7.2. The emulsion was spun for 30 minutes in an angle centrifuge, and the supernatant fluid following centrifugation was passed through a Berkefeld V filter. It was used within a short time after preparation.

Rabbit Erythrocytes.—To secure a suspension of red blood cells, a normal rabbit was bled from the heart under ether anesthesia; the blood was defibrinated, and the cells were washed twice with isotonic buffer solution or Ringer's solution. The top layer of cells was taken off after each washing in order to remove as many leucocytes as possible.

EXPERIMENTAL

In order to study the metabolism of a virus, it is necessary to have appreciable quantities of the active agent, free from living bacteria and viable tissue cells, suspended in small volumes of liquids. A suspension of the elementary bodies of vaccinia, prepared in the manner described, satisfies these requirements. In the following experiments we studied the consumption of oxygen and production of acid by washed elementary bodies of vaccinia under certain controlled conditions. Then, the effect of addition of freshly prepared tissue extracts containing a "respiratory supplement" to the suspension of elementary bodies was observed. Finally, the uptake of oxygen and production of acid by a mixture of elementary bodies and rabbit erythrocytes, under aerobic and anaerobic conditions, were measured.

In the first experiment the oxygen consumed by the elementary bodies was determined both before and after the addition of glucose, glucose monophosphate, and methylene blue, respectively.

Experiment 1.—A suspension containing 24 mg., dry weight, of elementary bodies free from living bacteria was prepared in isotonic buffer solution; the volume was then reduced to 1.6 cc. The elementary bodies were placed in a Warburg vessel; 0.1 cc. of 0.55 M glucose solution was put in one side arm and 0.1 cc. of glucose monophosphate solution in the other. Normal sodium hydroxide solution was placed in the inset vessel to absorb carbon dioxide. Both before and after addition of substrate, the amount of oxygen taken up during certain intervals of time was measured. Methylene blue was also added to the system and its effect noted. The results of the experiment are set forth in column 2 of Table I. In certain subsequent experiments similar observations on the amount of oxygen consumed by elementary bodies under the same conditions were made and the results of these experiments have been included in Table I for ease of comparison.

From the results shown in Table I it is obvious that there was a very small uptake of oxygen by the elementary bodies alone, most of which occurred in the first hour of observation; very little absorption followed the addition of glucose or glucose monophosphate; there was almost no effect from the addition of methylene blue to the system. These results differ markedly from those obtained with bacteria (12). The quantity of oxygen absorbed per milligram nitrogen, in the elementary bodies is much less (3 c.mm. compared with 15 to 340 c.mm.) than that taken up by resting bacteria (13). While the amount of oxygen consumed by bacteria is greatly increased by addition of glucose, this had no effect on the uptake of oxygen by elementary bodies. Furthermore, in the case of the elementary bodies the reaction is soon complete, and after a period of an hour or so no further absorption occurs, a phenomenon not noted in the case of bacteria observed for comparable periods of time. That the cessation of consumption of oxygen was not due to loss of activity of the virus was shown by inoculation of the virus into animals which revealed that the elementary bodies were as infectious at the completion of the experiments as they were at the beginning. The results obtained with elementary bodies differ also from those secured with spores. Goddard (14) has shown that the consumption of oxygen by dormant¹

¹The ascospores of the fungus *Neurospora* are normally dormant and will germinate only after they have been heated. The heat treatment which overcomes the dormancy consists in heating the spores to a temperature of 50°C. or higher for a few minutes and then cooling to room temperature. The spores germinate 3 to 5 hours after returning to the lower temperature.

TABLE I
Consumption of Oxygen by Elementary Bodies of *Vaccinia*

		Contents of vessel		
		cc.	cc.	cc.
24 mg. elementary bodies in 1.60 cc. phosphate buffer..		1.60		
11.5 " " " " 0.75 " " " ..			0.75	
8.0 " " " " 0.80 " " " ..				0.80
0.55 M glucose solution (in side arm)		0.10	0.10	0.20
0.10 M " monophosphate, potassium salt (in side arm)		0.10	0.10	0.20
0.001 M methylene blue (added after opening vessel) ...		0.10		
Substrate	Interval	Oxygen absorbed		
	min.	cm.m.	cm.m.	cm.m.
No substrate	8	3.6		
	21	7.2		
	42	8.2		
	60	9.2		
Glucose added from side arm	7			2.3
	10		2.6	
	40	1.0		
	45		4.1	
	60	0.0		
	63			2.9
	69		4.1	
	137			2.9
	180			2.9
Glucose monophosphate added from side arm	15		-0.5	
	22	-1.5		
	31		0.0	
	41	-0.5		
	60		1.0	
	65	0.5		
	105		0.0	
Methylene blue added after opening vessel	6	1.0		
	30	2.5		
	55	3.0		

Temperature, 37°C.; gas in vessel, air; NaOH in inset vessel.

The figures of column 3 are taken from column 2 of Table III, those of column 4 are taken from column 2 of Table IV.

spores of *Neurospora tetrasperma* is very slight. When the spores are activated, however, the amount of oxygen consumed is immediately greatly increased, and shows the same continuous character as that evidenced by bacteria.

Having found that the amount of oxygen absorbed by elementary bodies is very small, we next determined whether acid is produced by them under anaerobic conditions by measuring the amount of carbon dioxide released from a buffer solution containing sodium bicarbonate and carbon dioxide.

Experiment 2.—Elementary bodies (26.0 mg., dry weight) were washed several times in distilled water and finally suspended in 2.0 cc. of it. A dilute solution of sodium bicarbonate was added, and, after equilibrating with a definite partial pressure of carbon dioxide, measurements were made of the amounts of carbon dioxide liberated. After appropriate intervals of time glucose and later glucose monophosphate were added. The results of this experiment, and of other experiments done under similar conditions, are set forth in Table II.

The figures given in Table II show that a small amount of carbon dioxide was released, almost all of which was liberated in the first hour of observation. These results agree with those of the experiments in which absorption of oxygen was measured, and provide little or no evidence of a measurable amount of metabolism of elementary bodies under the conditions employed. In view of the obligate parasitism of viruses this result was not unexpected, since viruses evidently depend on host cells for certain essential factors. The suggestion was made that a fresh tissue extract might provide a more satisfactory substrate for the virus which would be suitable for the demonstration of its metabolism. It is known that extracts of many tissues contain a "respiratory supplement" which is capable of stimulating the consumption of oxygen of certain cells, such as erythrocytes (15). In order to learn whether the addition of tissue extracts containing such supplement to a suspension of elementary bodies would increase their metabolism to such an extent as to make it measurable, the following experiment was performed.

Experiment 3.—A quantity of elementary bodies was prepared, suspended in Ringer's solution, and divided into two equal portions. To one of these, 0.75 cc.

TABLE II

Production of Acid by Elementary Bodies of Vaccinia under Anaerobic Conditions

		Contents of vessel			
		cc.	cc.	cc.	cc.
26.0 mg. elementary bodies in 2.00 cc. distilled water..		2.00			
18.0 " " " " 0.90 cc. modified Ringer's solution.....			0.90		
10.2 mg. elementary bodies in 0.90 cc. modified Ringer's solution.....				0.90	
7.6 mg. elementary bodies in 0.70 cc. modified Ringer's solution.....					0.70
Modified Ringer's solution.....			0.90	0.90	0.60
0.31 M sodium bicarbonate.....		0.16	0.40	0.40	0.30
0.55 M glucose (in side arm).....		0.10	0.20	0.20	0.20
0.10 M glucose monophosphate, potassium salt.....		0.10			
Substrate	Interval	CO ₂ released			
	min.	cc. mm.	cc. mm.	cc. mm.	cc. mm.
No substrate	20	1.6			
	60	8.2			
Glucose added from side arm	6	-0.7		-2.5	
	22				1.3
	30	1.3			
	45	1.3	1.1		
	49				1.3
	68			-1.2	
	72	2.2			
	82				2.6
	95			0.0	
	109		3.3		
	125			-1.2	2.6
	155			-1.8	
	160		5.5		
Glucose monophosphate added from side arm	203			-0.6	
	212		11.1		
	19	0.7			
	38	2.6			
	56	3.3			

Temperature, 37.0°C.; gas in vessel, 5 per cent CO₂, 95 per cent N₂; pH 7.2.

The figures of column 3 are taken from column 2 of Table V.

of a tissue extract, prepared in the manner described above, was added; to the other an equal volume of a buffer solution was added. To test for the presence of a respiratory supplement in the extract two further mixtures were made, *viz.*, red blood cells plus buffer solution and red blood cells plus tissue extract. An additional control of tissue extract alone was run. The amounts of oxygen consumed by these preparations were measured, and the results are summarized in Table III.

TABLE III

Consumption of Oxygen by a Mixture of Elementary Bodies of Vaccinia and Tissue Extract, and by a Mixture of Rabbit Erythrocytes and Tissue Extract

		Contents of vessel				
		cc.	cc.	cc.	cc.	cc.
Elementary bodies in phosphate buffer solution, 23 mg. in 1.5 cc.....		0.75	0.75			
30% kidney extract.....			0.75	0.75		0.75
50% suspension rabbit red cells.....					0.75	0.75
0.066 M phosphate buffer solution pH 7.2.....		0.75		0.75	0.75	
0.55 M glucose (in side arm).....		0.10	0.10	0.10	0.10	0.10
0.15 M glucose monophosphate (in side arm).....		0.10	0.10	0.10	0.10	0.10

Substrate	Interval	Oxygen absorbed				
		min.	cc.mm.	cc.mm.	cc.mm.	cc.mm.
Glucose added from side arm immediately before beginning readings	10	2.6	4.1	1.1	4.2	5.7
	45	4.1	7.1	2.1	17.9	25.8
	69	4.1	9.4	4.3	23.9	34.1
Glucose monophosphate added from side arm	15	-0.5	-0.6	-0.5	3.6	7.7
	31	0.0	1.2	1.1	9.5	16.5
	60	1.0	3.5	3.2	20.3	31.0
	105	0.0	5.9	5.4	37.1	55.0

Temperature, 37.0°C.; gas in vessel, air; NaOH in inset vessel.

In Table III it will be seen that the amount of oxygen consumed by the mixture of elementary bodies and tissue extract is almost exactly the sum of that consumed by the two components of the mixture determined separately. On the other hand, the rate of consumption of oxygen by the red blood cells was definitely increased in the presence of the tissue extract, thus indicating that it contained an adequate amount of active respiratory supplement.

Since from the results of the experiment just described it appeared

that the addition of a respiratory supplement was without effect on the rate of consumption of oxygen by the elementary bodies, we decided to determine whether the elementary bodies carried substances capable of accelerating the metabolism of rabbit red blood cells.

Experiment 4.—In order to detect the presence in elementary bodies of a respiratory supplement-like substance the amount of oxygen consumed by a mixture of elementary bodies and red blood cells was determined, and compared

TABLE IV

Consumption of Oxygen by a Mixture of Elementary Bodies of Vaccinia and Rabbit Erythrocytes

		Contents of vessel		
		cc.	cc.	cc.
16 mg. elementary bodies in 1.6 cc. phosphate buffer solution.....		0.80	0.80	
50% rabbit erythrocytes.....			0.80	0.80
0.066 M phosphate buffer solution.....		0.80		0.80
0.55 M glucose (in side arm).....		0.20	0.20	0.20

Substrate	Interval	Oxygen absorbed		
	min.	c.mm.	c.mm.	c.mm.
Glucose added from side arm immediately before beginning readings	7	2.3	3.4	3.3
	63	2.9	5.7	4.9
	137	2.9	9.1	6.5
	180	2.9	10.8	8.7

Temperature, 37.0°C.; gas in vessel, air; NaOH in inset vessel.

with the amount of oxygen consumed by each separately in comparable periods of time. The elementary bodies and cells were suspended in 0.066 M phosphate buffer solution in the presence of a glucose substrate. The results are recorded in Table IV.

It will be seen from the results shown in Table IV that there was no greater consumption of oxygen by the mixture of elementary bodies and red blood cells than could be accounted for by the addition of the values obtained for identical quantities of each separately. Similar experiments were then performed under anaerobic conditions.

Experiments 5, 6, 7.—For the experiments in which the amount of acid produced by a mixture of red blood cells and elementary bodies under anaerobic

conditions was determined, the elementary bodies and red blood cells were suspended, after washing, in Ringer's solution which had been modified by the omission of sodium bicarbonate. The sodium bicarbonate was added at the beginning of the experiment. The results of one of the experiments are given in Table V.

In the first experiment in which the rate of production of acid by a mixture of elementary bodies and red blood cells was measured, the quantity of acid produced by the mixture was definitely greater than

TABLE V

Production of Acid by a Mixture of Elementary Bodies of Vaccinia and Rabbit Erythrocytes under Anaerobic Conditions

		Contents of vessel		
		cc.	cc.	cc.
36.0 mg. elementary bodies in 1.8 cc. modified Ringer's solution.....		0.90	0.90	
50% suspension rabbit erythrocytes.....			0.90	0.90
Modified Ringer's solution.....		0.90		0.90
0.31 M sodium bicarbonate solution.....		0.40	0.40	0.40
0.55 M glucose (in side arm).....		0.20	0.20	0.20
Substrate	Interval	CO ₂ released		
	min.	c.mm.	c.mm.	c.mm.
Glucose added from side arm immediately before beginning readings	45	1.1	37.8	41.1
	109	3.3	86.5	87.5
	160	5.5	121.4	122.4
	212	11.1	155.0	159.0

Temperature, 37.0°C.; gas in vessel, 5 per cent CO₂, 95 per cent N₂; pH 7.2.

that produced by either component measured separately. In order to confirm this finding, two additional experiments were performed. The results of these two agreed with each other; the data obtained in one of them are given in Table V, from which it will be seen that the rate of production of acid by the mixture was no greater than the sum of the rates of the two suspensions composing it. An acceptable explanation for the increased rate of production of acid manifested in the first of these three experiments is not apparent.

SUMMARY AND CONCLUSIONS

Previous investigations of the metabolism of viruses have been hindered by the difficulty or impossibility of securing adequate amounts of the active agents in a pure state. However, by the application of recently developed technics, it is possible to prepare large quantities of vaccine virus free from living host cells, and to concentrate the suspensions to any desired degree. Advantage has been taken of this in the present investigation. Large quantities of washed elementary bodies of vaccinia were prepared, and suspended in small volumes of liquid. The amounts of oxygen consumed aerobically and of acid produced anaerobically were measured, the latter as carbon dioxide released from a buffer solution containing sodium bicarbonate and carbon dioxide. Even when large amounts of virus were used (as much as 26 mg., dry weight, of elementary bodies) the quantities of oxygen consumed and of acid liberated were very small. Furthermore, the greater part of the gaseous exchange which occurred took place in the first hour of observation; during the succeeding periods no absorption of oxygen or liberation of carbon dioxide was demonstrated. No increased absorption followed the addition of glucose, glucose monophosphate, or methylene blue. At the conclusion of the experiments the virus was shown to be fully active. Such findings are in sharp contrast to the results to be expected if true respiration were taking place, as for example in resting bacteria, in which case the quantities of oxygen consumed are much greater and are relatively constant during the period of observation.

It was considered that the failure of elementary bodies to consume oxygen might be due to lack of a proper substrate, or of respiratory supplements. In an effort to supply these essentials, a tissue extract was prepared which was shown to contain respiratory supplements, and this was added to the suspension of elementary bodies. It had, however, no effect on the rate of utilization of oxygen by the elementary bodies.

Since elementary bodies alone, and in the presence of simple and complex substrates, showed no evidence of continued respiration, it was decided to ascertain whether they contained substances capable of stimulating the metabolism of other cells. Rabbit erythrocytes were

used for this purpose; and the amounts of oxygen consumed under aerobic conditions and of acid produced under anaerobic conditions, respectively, by the red blood cells were determined. In neither case was any consistent stimulation of metabolism demonstrated.

In the interpretation of the results of our experiments it must be borne in mind that the conditions under which they were performed are highly artificial, and while they are compatible with the survival of virus, there is no reason to suppose that they would permit its growth (3, 4). It may be said, however, that under the conditions which have been described above, no evidence was secured that vaccine virus, in considerable amounts, freed from viable host cells and bacteria, is capable of continued utilization of measurable quantities of oxygen, or of continued release of appreciable amounts of acid.

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THE BACTERICIDAL PROPERTIES OF ULTRAVIOLET IRRADIATED LIPIDS OF THE SKIN

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Many oils which normally will not kill bacteria are bactericidal after exposure to ultraviolet light or after ozonization (1-4). Not only are the irradiated oils bactericidal but their vapor inhibits bacterial growth and fogs photographic plates. These acquired properties are due to the oxygen attached to the molecules of the oil during irradiation or ozonization and subsequently released in the active state. That lipids of the skin acquire similar properties through irradiation is shown in the following experiments.

EXPERIMENTAL

Experiment 1.—The estimation of active oxygen in the lipids of irradiated dried skin.

(a) *Preparation of the Dried Skin.*—Skin from guinea pigs was dried so that oxidation of the lipids was prevented as far as possible. After the subcutaneous fat had been stripped from it the shaven skin removed from the abdomens and backs of guinea pigs was cut into small pieces. These fragments were distributed among several pyrex tubes which were immersed in a bath of butyl alcohol and solid carbon dioxide and attached to a vacuum pump. The skin was then dried *in vacuo* while frozen. The tubes were sealed while the vacuum was maintained and were not opened until the skin was needed for experimentation. The skin was removed from these animals immediately after they were killed, to avoid changes in the lipids from autolysis.

(b) *Experimental Procedure with Various Samples of Dried Skin.*—Six samples of the dried skin were used in these experiments. Sample A: This sample was accurately weighed immediately after it was removed from one of the sealed tubes. The weighed sample was extracted in 20 cc. of absolute methyl alcohol in the refrigerator for 24 hours. Sample B: A weighed sample was placed in the dark at 37°C. for 24 hours in a flat open dish, then extracted in methyl alcohol for 24 hours. All samples were shaken frequently and thoroughly during the extraction. Sample C: A quantity of skin freshly removed from a sealed tube was weighed, then suc-

cessively extracted with acetone, methyl alcohol and ether three times to remove the lipids. The lipid-free skin was dried and reweighed. 35 per cent of the weight was lost in the extraction. An accurately weighed sample of this extracted skin was placed in 20 cc. of methyl alcohol 24 hours. Sample D: An accurately weighed sample of dried skin removed from a vacuum tube was irradiated at 20 inches under a mercury quartz arc for 8 hours. The fragments of dry skin were stirred occasionally so that all surfaces of the fragments were exposed to the light. This sample was extracted in 20 cc. of methyl alcohol for 24 hours. Sample E: A sample of lipid-free skin similar to sample C was irradiated 8 hours and extracted in methyl alcohol. Sample F: A weighed sample of dried skin was irradiated 8 hours then extracted three times with acetone, alcohol and ether. The sample was first dried *in vacuo* and then further extracted in methyl alcohol (20 cc.)

(c) *Estimation of the Active Oxygen in the Samples of Skin.*—The active oxygen in the 24 hour methyl alcohol extracts of the various samples (A, B, C, D, E, and F) was determined by estimating colorimetrically the amount of ferrous iron oxidized to the ferric state by each extract. The reagent used (5) was prepared by adding 5 gm. of ammonium thiocyanate and 6 cc. of 6 N H_2SO_4 to 1000 cc. of absolute methyl alcohol. Immediately before the reagent was used it was saturated with ferrous ammonium sulfate by vigorous shaking with a few crystals of the salt. The red color developed on addition of this reagent to the methyl alcohol extracts was compared in a colorimeter with that developed in a series of standards containing 0.1, 0.05 and 0.01 mg. of ferric iron as ferric ammonium sulfate. 1 cc. of standard aqueous solutions containing the ferric iron was diluted to 10 cc. with methyl alcohol and 10 cc. of reagent were added. 10 cc. of the reagent were then added to 10 cc. of extracts A, B, C, E, F. The extract from sample D required dilution with methyl alcohol to bring it within the range of the standards. Readings were made 30 minutes after the addition of the reagent to the extracts and standard solutions. The reagent contained small amounts of ferric iron which varied slightly with each new solution. The necessary correction was made for each lot by calculating the amount of ferric iron in the reagent and adding this known quantity to both sides of the equation in determining the unknown amount of ferric iron in the methyl alcohol extracts. The data from one of several experiments have been assembled in Table I. In this table the calculated active oxygen (O_1) has been expressed in mg. per 100 gm. of dried lipid-containing skin. Samples weighed after the lipids were extracted were multiplied by 100/65 in calculating the active oxygen content (see preparation of sample C).

Experiment 2.—The effect of irradiation on the active oxygen content of lipids extracted from the skin. Skin was dried while frozen as in the preceding experiment, then successively extracted with acetone, alcohol and ether three times. The extracts were combined in a suction flask and the extracting reagents were evaporated at room temperature first by a water pressure suction pump, and finally by a mechanical high vacuum oil pump. Evacuation was continued until bubbles no longer formed when the flask was warmed to 50°C ., then the flask was sealed to maintain the vacuum and refrigerated until the lipid was used. Three

samples of this lipid warmed to 37°C., each slightly less than 0.2 cc. in volume, were transferred to flat bottomed dishes approximately 2 cm. in diameter. The samples were accurately weighed. One dish containing lipids (A) was placed in 20 cc. of methyl alcohol immediately. The others were irradiated with ultra-violet light, one (B) 4 hours and the other (C) 8 hours before they were placed in methyl alcohol. By warming the alcohol slightly most of the fat was dissolved and a fine suspension of the undissolved fat was obtained by shaking the tubes

TABLE I

The Effect of Irradiation on the Active Oxygen Content of Dry Skin

Sample	Weight	Lipids	Irradiation	Active O
	mg.		hrs.	mg per 100 gm.
A	680	Not extracted	None	4.1
B*	798	Not extracted	None	5.9
C†	529	Extracted	None	0.0
D	784	Not extracted	8	62.7
E†‡	546	Extracted	8	5.6
F§	795	Extracted	8	0.0

* Exposed to air 24 hours.

† Dry weight after lipid extraction.

‡ Lipids extracted before irradiation.

§ Lipids extracted after irradiation.

TABLE II

The Active Oxygen Content of the Irradiated Lipids

Sample	Weight	Irradiation	Active O
	mg	hrs.	mg per 100 gm.
A	156	None	25
B	198	4	84.7
C	210	8	88.5

vigorously. 1 cc. of each of these solutions was diluted ten times in methyl alcohol and 10 cc. of reagent (Experiment 1) were added to each of the diluted samples. The resultant color in each was compared with one of the series of standards used in the preceding experiment. The results of this experiment are found in Table II, in which the active oxygen is expressed in mg. per 100 gm. of lipid.

Experiment 3.—The fogging effect of irradiated lipids on photographic plates. Two small round flat bottomed dishes 1 cm. in depth and 2 cm. in diameter with ground lips were partially filled (0.5 cc.) with lipid extracted from the skin (Ex-

periment 2). The fat in one was exposed to ultraviolet light 8 hours, then both were covered with a photographic plate in a light-proof box at 37°C. After 24 hours the plate was developed. The vapor from the irradiated lipid fogged the plate intensely, while that from the normal lipid caused but faint fogging (Fig. 1).

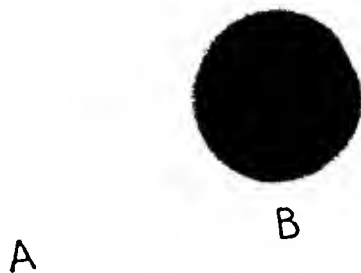


FIG. 1. The fogging effect of vapor from (A) non-irradiated and (B) irradiated lipid extracted from skin.

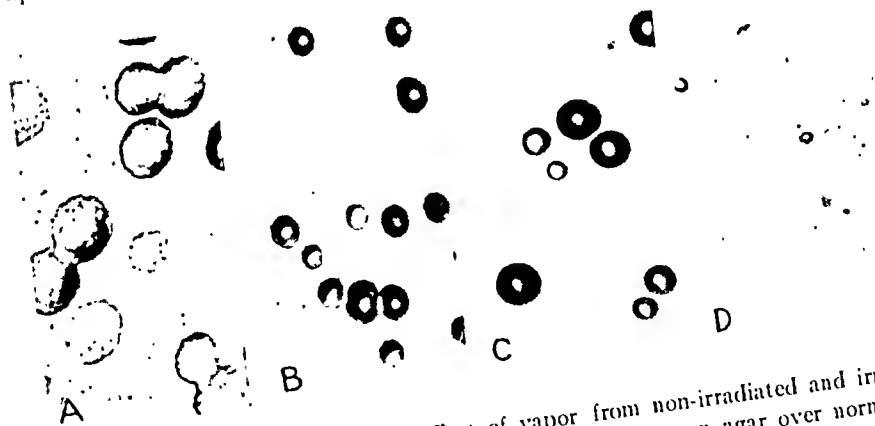


FIG. 2. The growth-inhibiting effect of vapor from non-irradiated and irradiated lipid. A, hemolytic streptococcus (H.S. 1) grown on agar over normal lipid. B, H.S. 1 grown over irradiated lipid. C, hemolytic streptococcus (H.S. 2) grown over normal lipid. D, H.S. 2 grown over irradiated lipid.

Experiment 4.—The growth-inhibiting effect of the vapor from irradiated lipids. The inhibiting effect of vapor from irradiated lipids has been compared with that of normal lipids. Two strains of hemolytic streptococcus, H.S. 1 and H.S. 2 were used. 18 hour cultures of these strains were diluted 1 to 5000 and

sprayed on a thin layer of cooled nutrient agar containing 0.1 per cent dextrose poured into small dishes 2 cm. in diameter and 1 cm. in depth. Experience had shown that a uniform distribution of colonies was obtained by the method of spraying employed. 0.2 cc. of lipid extracted from the skin was placed in each of four similar dishes. Two of these dishes containing lipid were exposed to ultraviolet light 8 hours and sprayed plate cultures of strains H.S. 1 and H.S. 2 were inverted over them. Similar sprayed plate cultures of H.S. 1 and H.S. 2 were inverted over dishes of normal lipid. The junctions of the dishes were then sealed and the cultures were incubated 24 hours. The inhibiting effect of the vapor from the irradiated lipids is shown in Fig. 2.

Experiment 5.—The bactericidal properties of irradiated lipids extracted from skin. The bactericidal properties of emulsions of irradiated lipids extracted from the skin have been compared with emulsions of normal lipids. Two strains of hemolytic streptococcus, H.S. 1 and H.S. 2, were used in these experiments.

TABLE III

The Bactericidal Properties of Emulsions of Irradiated Lipids Extracted from Skin

Bacterium	Irradiation	Duration of viability of hemolytic streptococcus				Final pH
		No cysteine	Cysteine 0.05 per cent	Cysteine 0.1 per cent	Cysteine 0.2 per cent	
H.S. 1	hrs.	hrs.	hrs.	hrs.	hrs.	
H.S. 1	None	D 79	L 104			6.8
H.S. 2	8	D 3	D 7			6.5
H.S. 2	None	D 70	L 104	D 10	D 33	6.8
H.S. 2	8	D 3	D 8	D 12	D 37	6.5

Each strain was set up with normal and irradiated lipid in the following manner. To each of a series of four tubes, the first containing 1 cc. of salt solution and the second, third and fourth tubes, 1 cc. of salt solution with neutralized cysteine HCl in concentrations of 0.05, 0.1 and 0.2 per cent respectively, was added 0.2 cc. of lipid which had been exposed to ultraviolet light 8 hours at 20 inches. 0.2 cc. of normal lipid was added to a control series of two tubes, the first containing 1 cc. of salt solution and the second 1 cc. of salt solution with a concentration of neutralized cysteine HCl of 0.05 per cent. These tubes were shaken in a machine 8 hours at room temperature, then the contents of each tube was poured on the centrifuged sediment from 1 cc. of an 18 hour culture of hemolytic streptococcus. Both strains of hemolytic streptococcus H.S. 1 and H.S. 2 were set up in this way. The emulsions of lipid and the suspended bacteria were then cultured at frequent intervals both in broth and on plates until the bacteria were dead. The results of these cultures have been arranged in Table III. In this table, D indicates that the bacteria were all dead and the hour at which the culture was made is shown by the numeral following the letter. L indicates that the bacteria

were still living and cultures were discontinued. The pH of these tubes was determined approximately by the colorimetric method after the experiment was finished. The pH ranged between 6.5 and 6.8.

SUMMARY AND CONCLUSIONS

The lipids of the skin after exposure to ultraviolet light are bactericidal. Since other fats and oils which have been irradiated are bactericidal on account of the active oxygen released on contact with bacteria, the mechanism of the bactericidal action of irradiated lipids of the skin must be similar because the lipids have the properties of other irradiated fats and oils. Irradiation increases the active oxygen content of dried skin markedly but little increase occurs if the lipids have been extracted. Although the normal lipids extracted from the skin contain some active oxygen, the active oxygen content is much increased by irradiation. The vapor from lipids exposed to ultraviolet light fogs photographic plates intensely and retards the growth of hemolytic streptococcus. When emulsified in salt solution, the irradiated lipids kill hemolytic streptococcus promptly in comparison with emulsions of lipid which have not been irradiated. The addition of neutralized cysteine HCl to the emulsions of the lipid, normal or irradiated, prolongs the life of bacteria suspended in the emulsions. This protective effect is due to the reducing action of the cysteine. Normal non-irradiated lipid, extracted from the skin under conditions which permit oxidation, kills bacteria more quickly than that used in these experiments, where precautions were taken to prevent oxidation (unpublished data). Even though these precautions were taken some oxidation occurred, because lipid so extracted contained some active oxygen, and bacteria lived longer in emulsions of this normal lipid if cysteine were added.

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VITAMIN C THERAPY AND PROPHYLAXIS IN EXPERIMENTAL POLIOMYELITIS*

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In previous experiments we have shown that multiple paralytic doses of poliomyelitis virus, when mixed with very small amounts of crystalline vitamin C (*l*-ascorbic acid), are rendered non-infectious as determined by intracerebral injection of such mixtures into *rhesus* monkeys (1). What made this phenomenon particularly interesting was the existence of a rather narrow quantitative range within which vitamin C seemed to be most effective, the optimal doses lying between 10 mg. and 1 mg. A similar zone was previously described by us in the inactivation of diphtheria toxin by vitamin C (2).

These observations naturally raised the question whether or not the injection of vitamin C into monkeys suffering from experimental poliomyelitis might be followed by any alteration in the severity of the infection. The experimental trial of this idea has led to encouraging results which have already been published in preliminary form (3). To summarize briefly: It was found that a small group of 4 monkeys which had received daily injections of 5 mg. of vitamin C, beginning with the day of the infection, survived without showing any symptoms of paralysis. In another group of 16 animals which had been treated with somewhat larger doses (50 to 100 mg.), 11 developed typical paralysis, 3 showed an atypical form of the disease (onset of paralysis delayed over 2 weeks), and 2 remained free from any paralytic symptoms. Finally, another group of 9 animals which had received very large doses of vitamin C (100 to 700 mg.) all succumbed to the disease in a typical manner. 10 control monkeys, injected

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intracerebrally like the treated animals with 0.1 cc. of a 10 per cent virus suspension, but left without treatment, all developed typical paralysis within from 6 to 11 days.

The limited data which formed the basis of this preliminary report naturally precluded the drawing of any definite conclusions. They seemed to suggest, however, that vitamin C when administered in the proper dose may possess distinct therapeutic properties in experimental poliomyelitis. It became necessary, therefore, to continue this line of work and to amplify our original observations with a larger number of tests. At the same time, it seemed advisable to investigate whether prophylaxis with vitamin C afforded any protection against subsequent inoculation with poliomyelitis virus. It is the object of this paper to present in detail the new experimental data obtained in the continued study of this problem, together with those reported previously in preliminary form.

EXPERIMENTAL WORK

The observations reported in this paper bear on a total of nearly 100 *rhesus* monkeys treated in various ways with vitamin C before or after intracerebral infection with poliomyelitis virus and about half that number of control animals which accompanied these tests. For the sake of clarity in presentation, these data will be arranged in three sections: the first dealing with therapeutic experiments; the second with prophylactic experiments; and the third describing experimental work of somewhat different nature, but generally related to the main problem under investigation.

1. Therapeutic Experiments

Rhesus monkeys weighing from 2000 to 3000 gm. were inoculated intracerebrally with three different amounts of poliomyelitis virus (Aycock strain), *i.e.*, 0.1 cc., 0.05 cc. and 0.01 cc. of a 10 per cent virus suspension. These animals were then treated for a period of 2 weeks with daily injections of vitamin C¹ of various dosage. 11 different series were run. In 9 of these, treatment was uniformly begun on the day of inoculation; in the last 2 series, the initial injection was delayed in some animals until 48 or 72 hours, respectively, after the day of infection.

¹ We are greatly indebted to Merck and Co., Rahway, New Jersey, for placing at our disposal a generous supply of cebione, their crystalline natural vitamin C preparation, in ampoules.

The doses of vitamin C covered a range from 700 mg. to 5 mg. and were mostly administered by the subcutaneous route. Control monkeys, which were infected intracerebrally at the same time with the corresponding dose of virus, accompanied each series. With the larger doses of virus we have satisfied ourselves, as a rule, with 2 controls for each series; when smaller doses of virus were employed, the number of controls was usually increased, in some cases to approximately equal the number of treated animals. All animals were carefully observed for 1 month and symptoms noted. In case of paralysis or death, an autopsy was made and the diagnosis confirmed by histological examination of the spinal cord. Surviving animals were reinoculated at the end of 1 month. The protocols of these individual series are given in Table I. A summary in which all animals are grouped according to the size of the infecting dose will be found in Table II, while Table III lists the results obtained in accordance with the amount of vitamin C injected.

A study of Table III, which considers only the effect of various dosages of vitamin C, without regard for the varying severity of the infection, leaves no doubt that large doses of vitamin C, *i.e.*, amounts ranging from 700 to 100 mg., had no influence whatsoever on the course of the disease. Thus, we find that all 10 monkeys which had been treated with such excessive amounts succumbed to the infection. On the other hand, animals treated with intermediate doses (50 to 10 mg.) occasionally remained free from paralytic symptoms or else developed paralysis only after prolonged incubation periods. This is illustrated by the fact that of a total of 19 monkeys in this category, 3 escaped the disease entirely and 3 came down with paralysis between 15 and 21 days, the remaining 13 animals developing typical poliomyelitis. More favorable results, however, were obtained with monkeys which had received 5 mg. of vitamin C. To wit, of a total of 33 animals treated with this particular dose, not less than 16 survived without showing any evidence of paralysis, 2 developed the disease after 15 and 16 days, respectively, while the remainder, *i.e.*, 15, succumbed to typical poliomyelitis.

These results must be evaluated, of course, in the light of the incidence of paralysis among the controls. If we consider the controls first *en bloc*, irrespective of differences in the size of the infecting dose, it appears that of a total of 38 control monkeys, 36 succumbed to the disease (34 developing typical poliomyelitis within less than 2 weeks incubation period and 2 after 17 and 16 days, respectively), while 2 failed to show any manifest symptoms of paralysis.

Directly comparable data may be obtained from a study of Table II in which all animals, treated and untreated, are listed according to the size of the infecting dose. It will be seen that of a total of 34 vitamin C-treated animals which had been infected with 0.1 cc. of virus, 25 developed typical and 3 atypical poliomyelitis, while 6 remained free from any paralytic symptoms. All 10 controls infected with the same dose of virus came down with typical poliomyelitis. Of 6 treated animals infected with half the amount of virus, *i.e.*, 0.05 cc., 1 escaped the disease, 2 developed atypical and 3 typical poliomyelitis. Again, all 7 controls infected with the same dose of virus developed the disease, 6 typically and

TABLE I
Vitamin C Therapy in Experimental Poliomyelitis

	Monkey	Dose of virus	Dose of vitamin C	Result	Controls
		cc.	mg.		
Series 1	O83	0.1	500	Complete paralysis, 14 days	O69 Complete paralysis, 8 days
	O70	"	200	" " 8 "	O90 " " " "
Series 2	Q28	"	700	" " " "	
	Q29	"	"	" " " "	
	Q30	"	500	" " 5 "	
	Q31	"	"	" " 7 "	Q13 Complete paralysis, 8 days
	Q32	"	200	" " " "	Q26 Partial paralysis, 9 "
	Q33	"	50	Partial paralysis, 11 "	
	Q34	"	"	Complete paralysis, 5 "	
Series 3	Q48	"	500	" " 7 "	
	Q47	"	100	" " " "	
	Q46	"	50	" " 10 "	
	Q49	"	"	" " 8 "	Q54 Complete paralysis, 11 days
	Q50	"	"	" " 11 "	Q55 " " " "
	Q53	"	"	" " 21 "	Q90 " " 9 "
	Q51	"	25	" " 10 "	Q76 " " 11 "
	Q52	"	"	" " 7 "	
	Q45	"	"	No paralysis	
	Q78	"	"	Complete paralysis, 16 days	
Series 4	R13	"	"	" " 11 "	
	R15	"	10	" " 8 "	
	R16	"	"	" " 15 "	R12 Complete paralysis, 7 days
	R17	"	5	No paralysis	
	R18	"	"	" "	
Series 5	R19	"	25	" "	
	R20	"	10	Complete paralysis, 11 days	R25 Complete paralysis, 7 days
	R21	"	"	" " " "	R37 " " " "
	R22	"	5	No paralysis	
	R23	"	"	" "	
Series 6	S21	"	"	Complete paralysis, 7 days	S18 Partial paralysis, 8 days
	S25	"	"	" " 6 "	S20 Complete paralysis, 6 "
	S48	"	"	" " " "	R49 " " 14 "
	R51	"	"	" " 9 "	R50 " " 8 "
	R53	"	"	Partial paralysis, 8 "	R52 " " " "
	"	"	"		R60 " " 6 "
	"	"	"		R63 " " 9 "
	"	"	"		R3 " " 5 "

TABLE I—*Concluded*

	Monkey	Dose of virus	Dosage of vitamin C	Result	Controls
		cc.	mg.		
Series 7	R89	0.05	5	Complete paralysis, 8 days	R95 Complete paralysis 8 days
	S5	"	"	" " 9 "	R91 " " 7 "
	"	"	"	"	R98 " " 11 "
	"	"	"	"	R54 " " 17 "
Series 8	S78	"	25	Complete paralysis, 9 days	S67 " " 12 "
	S75	"	5	" " 16 "	S68 " " " "
	S88	"	"	No paralysis	S94 " " 11 "
	S89	"	"	Partial paralysis, 15 days	
Series 9	S90	0.01	100	Complete paralysis, 9 "	
	S91	"	50	" " 10 "	S69 " " 7 days
	S79	"	25	No paralysis	S70 " " " "
	S76	"	5	" "	
Series 10	S95	"	"	Complete paralysis, 8 days	T1 Complete paralysis, 16 days
	S96	"	"	No paralysis	T2 " " 7 "
	S97	"	"	" "	T3 " " 8 "
	S98*	"	"	Complete paralysis, 9 days	T5 " " 7 "
	S99*	"	"	" " 8 "	T6 " " 9 "
	S100*	"	"	" " 9 "	T7 No paralysis
	"	"	"	"	T9 Complete paralysis, 7 days
Series 11	T18	"	"	Complete paralysis, 9 days	
	T19	"	"	" " 8 "	
	T20	"	"	No paralysis	
	T21	"	"	" "	
	T22	"	"	" "	
	T23	"	"	" "	T15 No paralysis
	T25**	"	"	" "	T16 Complete paralysis, 9 days
	T26**	"	"	" "	T17 " " " "
	T27**	"	"	" "	
	T28**	"	"	Complete paralysis, 9 days	
	T29**	"	"	No paralysis	
	T30**	"	"	Complete paralysis, 9 days	

All treated animals received daily injections of vitamin C of the indicated dosage for a period of 2 weeks, or until the onset of paralysis. Treatment was begun on the day of infection, excepting animals marked ** in which the first injection was not given until 48 hours after infection, and animals marked * in which the first injection was not given until 72 hours after infection.

1 after 17 days. More significant results were obtained with monkeys which had been infected with the smallest dose of virus. Of 22 vitamin C-treated animals,

TABLE II
Effect of Various Doses of Vitamin C in Experimental Poliomyelitis with Respect to Variations in the Size of the Infecting Dose of Virus

<i>Treated Animals</i>					
Dose of virus	Dosage of vitamin C	Number of monkeys	No paralysis	Atypical paralysis	Typical paralysis
cc. 0.1	700-100	9	0	0	9
	50- 10	16	2	3	11
	5	9	4	0	5
		34	6	3	25
0.05	700-100	0	0	0	0
	50- 10	1	0	0	1
	5	5	1	2	2
		6	1	2	3
0.01	700-100	1	0	0	1
	50- 10	2	1	0	1
	5	19	11	0	8
		22	12	0	10
		62	19	5	38

<i>Untreated Control Animals</i>				
Dose of virus	Number of monkeys	No paralysis	Atypical paralysis	Typical paralysis
cc. 0.1	19	0	0	19
0.05	7	0	1	6
0.01	12	2	1	9
	38	2	2	34

Atypical paralysis = onset of paralysis later than 2 weeks following infection.

Typical paralysis = onset of paralysis within 2 weeks following infection.

which had been infected with 0.01 cc. of virus, 12 remained free from any paralysis, while 10 came down with typical poliomyelitis. In contrast herewith, we find among 12 control animals infected with the same amount of virus, 10 which suc-

cumbed to the disease (9 typically and 1 after 16 days) and only 2 that failed to show any paralysis.

While it can readily be appreciated that the chances for a therapeutic effect must improve with a reduction in the size of the infecting dose, we believe that even animals infected with the larger amounts of virus show evidence of having benefited from the treatment, provided the dosage of vitamin C is kept within optimal limits. Thus, we have among a group of 14 monkeys infected with 0.1 cc. or 0.05 cc. of virus, which were treated with 5 mg. of vitamin C beginning with the day of the inoculation, no less than 5 survivors without paralysis, whereas all 26 controls infected with the same amounts of virus developed the disease. In a similar way, considerably better figures are obtained for monkeys which had been infected with the smallest amount of virus, *i.e.*, 0.01 cc., if we exclude animals treated with the larger doses and consider only those which had received 5 mg. of vitamin C. It will be found that of 10 such animals, in which treatment was begun on the day of infection, 7 escaped the disease. Even under adverse conditions, when treatment was delayed until 48 or 72 hours after infection, this method of treatment has apparently saved 4 of 9 monkeys from paralysis. When left without treatment, only 2 of 12 controls inoculated with the same dose of virus, *i.e.*, 0.01 cc., remained free from paralysis.

Whether the data are taken as a whole or are interpreted according to gradations in the size of the infecting dose, it appears that treatment with 5 mg. of vitamin C has reduced the incidence of paralysis roughly by one-half as compared with untreated controls. Thus, we have among a total of 33 monkeys which were treated with this dose, 51.5 per cent (17) that developed paralysis against 94.7 per cent (36) among a total of 38 controls. Similarly, among 19 of these treated animals, which had been infected with 0.01 cc. of virus, the incidence of paralysis stands at 42.1 per cent (8) as compared with 83.3 per cent (10) among 12 corresponding controls. While the above figures would seem to be definitely significant, we do not wish to overemphasize their importance, because of the limited number of controls inoculated with the smallest dose of virus. Moreover, a study of the individual series makes it clear that distinct limitations are set in the extent and regularity of the therapeutic effect by experimental factors that are beyond control. All attempts to improve upon these results by other modifications in the form of treatment—*i.e.*, further reduction in the dosage of ascorbic acid to 1 mg. and less; gradual increases from day to day; intraspinal injection of this substance, or combination of vitamin C administration with the injection

of cortin—have been without avail. It is possible that a greater percentage of treated animals might survive after intranasal instillation of the virus, but this method of infection would automatically increase the number of survivors among the controls without necessarily strengthening the statistical significance of the results.

It is a common experience that the infectivity of passage strains of virus may fluctuate considerably from time to time (4). But even at times of maximum virulence an occasional monkey may survive without paralysis following intracerebral injection of an amount of virus which is capable of producing prostrating paralysis or death in all other animals in that particular series. Obviously, such irregularities occur more frequently with the smaller doses of virus al-

TABLE III
Comparison of the Effect of Various Dosages of Vitamin C

Dosage of vitamin C	Number of animals	No paralysis	Atypical paralysis	Typical paralysis
cc.				
700-100	10	0	0	10
50- 10	19	3	3	13
5	33	16	2	15
	62	19	5	38

Explanation for atypical and typical paralysis see in Table II.

though they are not at all uncommon with animals given massive doses. As regards the particular strain of virus employed in this work, we have observed over a period of about 7 years among several hundred control animals between 5 and 10 per cent that have failed to develop paralysis upon intracerebral injection of doses of virus ranging from 1 cc. to 0.01 cc. of a 10 per cent virus suspension. Moreover, when paralysis occurred, it was usually so severe that the animal became completely prostrated. Repeated titrations of our strain during the year when these experiments were under way indicate that the minimum paralytic dose of virus at that time was well below 0.01 cc. (see Table IV). The reasons for the occasional survival without paralysis of control animals injected intracerebrally with multiples of the minimum paralytic dose of virus are not clear. They

are just as obscure as the more pronounced variations in the susceptibility of monkeys observed with methods of infection other than intracerebral injection of the virus (intranasal, subcutaneous, intradermal) or the widespread insusceptibility among other animal species, including man. Suffice it to say that technical errors can usually be eliminated and that such animals evidently possess an exceptionally high degree of natural resistance. This is also suggested by the fact that the resistant animals often refuse to develop

TABLE IV

Titration of Infectivity of Aycock Strain of Poliomyelitis Virus during 1935 (Intracerebral Injection)

Monkey	10% virus suspension	Result
	cc.	
N83	0.1	Complete paralysis, 7 days
N84	"	" " 8 "
N85	"	" " 5 "
N86	0.05	" " 9 "
N87	"	" " 6 "
N100	"	" " 5 "
O1	0.01	" " " "
O2	0.005	" " " "
O3	0.001	Questionable paralysis
O20	0.1	Complete paralysis 7 days
O21	"	" " 5 "
O22	0.01	" " 9 "
O23	"	Partial paralysis, 17 "

the disease on reinfection. In order to determine to what extent such refractory animals may have been present among our treated animals and controls, we have reinoculated all surviving animals at the end of the 1 month period of observation.

It appears from Table V that of 15 treated animals which had escaped paralysis and which had not died of intercurrent disease before 1 month had elapsed, all but 1 developed typical poliomyelitis upon intracerebral reinjection with 0.1 cc. or 0.01 cc. of virus. Of the 2 surviving controls, on the other hand, 1 remained refractory while

the other developed typical poliomyelitis. This result, in our opinion, suggests that practically all of our treated animals were potentially susceptible to the virus and that, if any escaped paralysis during the first infection, the escape may well have been due to the vitamin C treatment. It also serves to illustrate that a non-paralyzing infection

TABLE V
Reinfection of Surviving Monkeys, Treated and Untreated

Mon-key	Type of animal	Dose of virus	Result	Controls
R17	Treated	0.1	Complete paralysis, 7 days	S18 Partial paralysis, 8 days
R19	"	"	Partial paralysis, 11 "	S20 Complete paralysis, 6 "
R22	"	"	Complete paralysis, 6 "	R3 " " 5 "
R23	"	"	" " 9 "	R49 " " 14 "
		"		R50 " " 8 "
		"		R52 " " " "
		"		R60 " " 6 "
		"		R63 " " 9 "
S76	Treated	0.01	Complete paralysis, 10 days	
S79	"	"	" " 11 "	T12 Complete paralysis, 8 days
S96	"	"	" " 8 "	
S97	"	"	" " 7 "	
T7	Untreated	"	No paralysis	
T20	Treated	"	Complete paralysis, 5 days	
T21	"	"	Partial paralysis, 11 "	
T23	"	"	Complete paralysis, 7 "	T31 Complete paralysis, 8 days
T25	"	"	Partial paralysis, 11 "	
T26	"	"	Complete paralysis, 9 "	
T27	"	"	No paralysis	
T29	"	"	Partial paralysis, 11 days	
T15	Untreated	"	Complete paralysis, 9 "	

with poliomyelitis virus rarely if ever is followed by immunity in the monkey, a fact which we have amply demonstrated in a previous paper (5).

2. Prophylactic Experiments

In view of the fact that therapeutic injection of vitamin C in proper doses seemed to have a favorable effect on the course of the established disease, we have

next investigated whether a prophylactic administration of this substance afforded any protection against subsequent inoculation. To achieve this end, two different methods were followed. A total of 16 monkeys were prepared by daily injections of vitamin C of various dosage (25 mg. to 500 mg.) for a period of 1 or 2 weeks and then injected intracerebrally with 0.1 cc. of virus; at the same time, 8 control animals were injected intracerebrally with the identical dose of virus. A second group of 10 monkeys were given daily, for a period of 2 weeks, approximately 150 mg. of vitamin C in their food. This amount represents merely a rough estimate since the eating habits of monkeys make it impossible to obtain control over the amount actually ingested. At the end of the 2 weeks period, these monkeys were injected intracerebrally with 0.01 cc. of virus; 7 control animals injected with the same dose of virus accompanying this test. The results of these two experiments appear in Table VI.

As may be seen from Table VI, we have in the first experiment only one monkey which survived without paralysis, the remaining 15 animals all developing the disease like the corresponding 8 controls. In the second experiment (feeding) we find among 10 prepared animals again one survivor without paralysis and 2 additional animals that developed the disease after prolonged incubation periods (18 and 19 days, respectively). This result, however, is offset by the fact that of 7 corresponding control animals, one survived without paralysis and another one developed the disease only on the 15th day. We conclude, therefore, under the conditions of the test, that prophylaxis with vitamin C was without any significant effect on the course of subsequent infection with poliomyelitis virus.

3. Miscellaneous Experiments

In this section we have grouped together some miscellaneous experiments which are not strictly concerned with vitamin C prophylaxis or therapy but which are nevertheless intimately related to the main problem under investigation.

The first question we were interested in was whether the apparent therapeutic effect of vitamin C could be ascribed to any direct action of the ascorbic acid on the virus. An answer to this question could be found by determining whether or not vitamin C, when injected intracerebrally in a single dose at the site of infection, was capable of preventing the disease. Accordingly, a group of 4 monkeys was injected intracerebrally with 5 mg. and 10 mg. of vitamin C, respectively. After 2 hours had elapsed, a dose of 0.05 cc. of virus was injected through the same puncture in the skull into the brain. Another group of 4 monkeys was treated in the same way, except that here the virus injection preceded the injection of the drug by 2 hours. All 8 experimental animals developed paralysis as did all of the 9 controls which had been infected with the same dose of virus. It follows from this experiment that vitamin C, although seemingly virucidal *in vitro*, fails to accomplish a local sterilizing effect in the central nervous system, under the conditions of our test.

TABLE VI
Attempts at Vitamin C Prophylaxis in Experimental Poliomyelitis

Monkey	Preparation	Dose of vitamin C	Dose of virus	Result	Controls
Experiment 1	1 wk. injection	mg.	cc.		
O63		200	0.1	Complete paralysis, 9 days	O62 Complete paralysis, 7 days
O74	" "	50	"	Partial paralysis, 11 "	O90 " " 8 "
O75	" "	"	"	Complete paralysis, 9 "	O69 " " " "
Q36	" "	25	"	" " " "	
Q37	" "	"	"	" " " "	
Q39	" "	50	"	" " 7 "	
Q40	" "	"	"	" " 13 "	
Q41	" "	"	"	" " 11 "	Q34 Complete paralysis, 11 days
Q42	" "	100	"	Partial paralysis, " "	Q55 " " " "
Q44	" "	500	"	" " 12 "	Q90 " " 9 "
				Complete paralysis, 7 "	Q76 " " 11 "
Q69	2 wks.	100	"	" " 10 "	
Q70	" "	"	"	" " 8 "	
Q71	" "	50	"	No paralysis	
Q72	" "	"	"	Complete paralysis, 7 days	Q87 Partial paralysis, 16 days
Q73	" "	25	"	" " 8 "	
Q74	" "	"	"	" " " "	
Experiment 2	2 wks. feeding		0.01		
S30		150		Partial paralysis, 18 "	T1 Complete paralysis, 16 days
S31	" "	"	"	Complete paralysis, 7 "	T2 " " 7 "
S32	" "	"	"	" " 11 "	T3 " " 8 "
S33	" "	"	"	" " 7 "	T5 " " 7 "
S34	" "	"	"	No paralysis	T6 " " 9 "
S36	" "	"	"	Complete paralysis, 19 days	T7 No paralysis
S37	" "	"	"	Partial paralysis, 9 "	
S39	" "	"	"	Complete paralysis, 10 "	T9 Complete paralysis, 7 days
S40	" "	"	"	" " 14 "	
				" " 9 "	

Experiments were next carried out to determine whether the addition of vitamin C to normal, non-neutralizing monkey serum would render the serum virucidal. While such "vitaminized" sera have occasionally brought about neutralization of the virus in mixture tests, irregular and confusing results were obtained on repetition with different doses of vitamin C. This makes it difficult to delimit clearly a neutralizing zone that might be compared with the range of natural vitamin C content of serum. Again, we have been unable up to the present to determine that the serum of normal monkeys, which had received large doses of vitamin C, acquires the property of neutralizing the virus as the result of such preparation. It seems as if inactivation of the virus by vitamin C in the presence of serum is by no means as regular and clear cut a phenomenon as its inactivation by the same substance in aqueous solution. This phase of the work is being continued.

We should finally mention in passing a short set of experiments designed to test the specificity of the apparent therapeutic effect of vitamin C. This could best be done by determining whether the use of other vitamins had any beneficial effect on the course of the disease. 4 monkeys were injected intracerebrally with 0.1 cc. of virus. 2 were given daily injections of 100 mg. of vitamin A (in the form of crystalline carotene), the 2 other animals receiving instead daily injections of 1 gm. of vitamin B (in the form of powdered yeast). All 4 monkeys developed typical poliomyelitis as did 4 accompanying controls. Apparently therefore, under the conditions of our test, vitamins A and B were without therapeutic effect in experimental poliomyelitis.

DISCUSSION

For a number of years we have contended that success in the control of infantile paralysis must depend upon the accumulation of more precise knowledge regarding the mechanism of natural protection against this disease (6). Once it is clearly understood why the majority of children and an even greater number of adults fail to develop paralysis upon exposure to the infectious agent, or—and this is expressing the same thought perhaps more trenchantly—why certain exceptional children are so highly susceptible that first contact with the virus leads to invasion of the central nervous system, it might be possible to apply the same principles to the therapy and prophylaxis of the disease.

Protection in poliomyelitis occurs in the form of two basic types of resistance which we believe to differ fundamentally from each other. The first is found in the solid immunity which is acquired after recovery from a paralyzing attack, a form of protection essentially due to a refractory state of the nerve tissue, which may or may not be accompanied by the presence of circulating antibodies. This protection, specific in character, nearly absolute in intensity and permanent in duration, in our opinion follows only after contact of the susceptible nerve cells with living virus, its development depending upon the production of actual lesions. The second, and as it seems to us more important type of protection, is represented by the resistance of naturally insusceptible individuals to this extraordinarily selective disease. Such resistance is expressed by complete insusceptibility of certain animal species, by the varying susceptibility of different human races, or in individual variations within the same race. The extensity and intensity of this non-specific resistance is characteristically conditioned by innate and environmental factors, such as heredity, sex, age, season and locality.

Two opposing theories have been invoked to explain the mechanism of this natural resistance, particularly in the case of man. The first, supported mainly by indirect epidemiological deduction, interprets the phenomenon as due to well nigh universal immunizing contact with the virus, progressing with the advance of age. It is assumed to be brought about by strictly subclinical immunization or by manifest but abortive attacks. According to the second theory, poliomyelitis is essentially a developmental disorder of youth, and protection against the disease is chiefly due to physiological factors. The fundamental difference in these two concepts is perhaps more clearly illustrated by their implications. The immunization theory regards all human beings as primarily susceptible and attributes protection exclusively to acquired specific immunity; hence, cases must develop either in individuals that have escaped such immunizing contact or else in persons that are less immunizable. The so called maturation theory, on the other hand, holds that man, under natural conditions of infection, is normally insusceptible and can carry the virus with impunity. Production of the disease which occurs only in comparatively few individuals, in spite of widespread chances for infection,

must therefore be brought about by some transient physiological abnormality or deficiency, hormonal or nutritional in character.

It has been our conviction that the known facts agree better with the theory of physiological resistance than with the assumption of an almost universal latent immunization. The reasons for this belief are founded briefly (*a*) on the inability to demonstrate clearly a process of subclinical immunization in either the monkey or man; (*b*) the lack of evidence that virucidal function of the serum and bodily resistance to infection in man or animals are necessarily correlated with previous exposure to the virus, and (*c*) direct experimental data which indicate that poliocidal substances found in normal human or animal tissues, serum or other body fluids are probably not of antibody character but resemble agents of vitamin-like or hormonal nature (7).

We have reviewed this controversy since on its determination depends any reasonable approach to treatment and prevention of poliomyelitis. Specific means of therapy through convalescent or immune serum having failed, experimentally as well as clinically, attention has concentrated lately on specific prophylaxis. Two methods of active immunization have been proposed, one involving the use of killed, the other the use of live virus. As might have been predicted, both have been given up. It appears that immunization with killed virus, although engendering antibody formation, falls short of providing cellular protection which is the prominent characteristic of true immunity in poliomyelitis (8). The use of live virus, on the other hand, even though attenuated, involves too great a risk in human beings. The chances, moreover, are that naturally insusceptible individuals will destroy the virus without deriving any more benefit from it than from casual contact, and that susceptible individuals may develop the disease (9). It is inevitable, therefore, that logical prophylaxis and treatment of infantile paralysis must center around attempts to imitate the mechanism of natural defense by enhancing normally functioning non-specific agencies, or by correcting the existing physiological deficiency by providing the missing nutritional or hormonal elements in adequate amounts and proportions. It seems from the results of our work that this might possibly be accomplished by supplying the infected individual with an optimum amount of vitamin C.

The assumption that vitamin C is an important factor of non-specific defense in infantile paralysis is suggested not only by the fact that ascorbic acid behaves like an unusually potent virucidal agent *in vitro*, but is also supported by certain other considerations. Thus, vitamin C is found in all normal body fluids which possess poliocidal properties (serum, tears, placenta, pregnancy urine, adrenal extracts). Next to the adrenal glands, it is present most abundantly in the central nervous system (10). During pregnancy and lactation its concentration in the body seems to be sharply increased, the placenta and milk representing the principal sources of exogenous supply for the embryo and newborn during prenatal and postnatal life (11). It is assumed by some investigators (12) that infants during the first few months after birth are capable of synthesizing this substance, like the rat, but that this capacity is lost or greatly diminished at the time of the physiological involution of the adrenal cortex, which occurs towards the end of the 1st year. These two points in the development of the child coincide characteristically with the maxima and minima of susceptibility to poliomyelitis.² Little is known about the actual demand for vitamin C, the extent of its assimilation and rate of excretion in health and disease and under varying environmental conditions. Suffice it to say, that although the precise relationship between the glands of internal secretion and vitamin metabolism is as yet not well understood, it is becoming increasingly clear that their operation is intimately interlocked since fluctuations in the supply of the latter lead to dysfunction of the former. Even though fully developed *C avitaminosis*, long recognized as the classical cause of scurvy, is but rarely observed today under normal living conditions, recent experience has shown that examples of minor deficiencies, or *C hypovitaminoses*, are by no means infrequent (13). The so called prescorbutic state, for instance, is not uncommonly found in actively growing children whose vitamin C requirements are known to exceed greatly those of adults for the maintenance of an adequate level. That this level may change considerably from time to time, possibly through changes in the thyroid gland (14), is suggested by seasonal fluctuations in the content of vitamin C in cows' milk (15) and in the rate of

² A similar phenomenon may be observed in diphtheria.

its excretion in man through the urine, as determined by tolerance tests (16). A serious disturbance of vitamin C storage is furthermore indicated during poliomyelitic infection by the occurrence of severe lesions in the adrenal gland (17) and an actual loss of reducing substances in this organ (18).³ To this must be added the pathognomonic frequency in infantile paralysis of gastrointestinal disorders—so often encountered in children suffering from a low grade C hypovitaminosis—which in turn lead to a further impoverishment of the vitamin C reserves of the body; this may be due, in part at least, to the vitamin-splitting properties of certain intestinal strains of *B. coli*, as suggested by the work of Stepp and Schroeder (21). A vicious circle might thus be formed which would lower resistance in certain individuals at certain times to a critical point and permit systemic invasion of the ubiquitous virus, thus serving to reconcile the constitutional peculiarities of the poliomyelitic child (22) with the epidemiological vagaries of the disease.

There are, however, some contradictory experimental facts that must not be ignored. First, we have been unable to obtain neutralization of the virus *in vitro* with normal monkey brain in spite of its presumably high vitamin C content (23). The same is true for adrenal tissue, although its three principal physiological constituents, *i.e.*, adrenalin, cortin and ascorbic acid, in isolated form, have proved highly virucidal. Second, the concentration of vitamin C in the tissues is said to diminish with the advance of age (24), while the reverse is observed with respect to the neutralization phenomenon by serum. Third, vitamin C-deficient guinea pigs are still refractory to poliomyelitic infection (25). These questions need further elucidation as does the problem of how vitamin C accomplishes its apparent therapeutic effect in experimental poliomyelitis, whether through direct action on the virus, or by stimulating certain enzyme systems, or by changing cell permeability. It is noteworthy that ascorbic acid, unlike immune serum, fails to display any preventive effect in the monkey while there are reasons for believing that it may be effective in therapy. This would seem to indicate that the substance operates only when actually

³ Again it should be pointed out that similar observations have been made in diphtheria (19) which seems to share certain aspects of its susceptibility problem with infantile paralysis (20).

needed, and that vitamin C upon injection into normal, non-deficient animals is not retained but rapidly eliminated. However, different results might be obtained in human prophylaxis, provided that further research reveals a causal relationship between disposition to infantile paralysis and faulty vitamin C metabolism.

SUMMARY AND CONCLUSIONS

1. A group of 34 monkeys were infected intracerebrally with 0.1 cc. of a 10 per cent virus suspension. Following infection, 9 animals were treated with daily injections of 700 to 100 mg., 16 with 50 to 10 mg. and 9 with 5 mg. of vitamin C for a period of 2 weeks. In the whole group there were 6 animals that survived without showing any evidence of paralysis. 2 of these had received 50 to 10 mg. while 4 had received 5 mg. All of 19 untreated control monkeys, infected simultaneously with the same amount of virus, developed paralysis.

2. Another group of 6 monkeys were infected intracerebrally with 0.05 cc. of virus. Following infection, one animal was treated in the same manner with 25 mg. and 5 with 5 mg. of vitamin C. In the whole group there was one animal that survived without showing any evidence of paralysis. This animal had received 5 mg. All of 7 untreated control monkeys, infected simultaneously with the same amount of virus, developed paralysis.

3. A third group of 22 monkeys were infected intracerebrally with 0.01 cc. of virus. Following infection, one animal was treated in the same manner with 100 mg., 2 with 50 to 10 mg., and 19 with 5 mg. of vitamin C. In the whole group there were 12 animals that survived without showing any evidence of paralysis. One of these had received 10 mg., while 11 had received 5 mg. Of 12 untreated control monkeys, infected simultaneously with the same amount of virus, 2 failed to show any paralytic symptoms and 10 developed paralysis.

4. A summary of the results obtained in all three groups shows: (a) that among a total of 62 treated monkeys, 19 survived without paralysis and 43 succumbed to the disease, while of a total of 38 untreated controls, only 2 failed to develop paralysis and 36 succumbed to the disease; (b) that treatment with large doses of vitamin C was without any beneficial effect (all 10 monkeys which had received 700

to 100 mg. developing paralysis), that the administration of intermediate doses was followed by occasional survival without paralysis of the treated animal (3 monkeys surviving of a total of 19 which had received 50 to 10 mg.), and that nearly one-half of the animals which had received small doses escaped the disease (16 monkeys surviving of a total of 33 which had received 5 mg.).

5. Attempts to protect monkeys against subsequent intracerebral infection by the prophylactic administration of vitamin C, either *per os* or parenterally, have produced negative results.

6. The pathogenesis of infantile paralysis is discussed in the light of the experimental findings and the possibility is suggested that vitamin C represents one of the deficiency factors in the susceptibility problem of the human disease.

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THE BACTERICIDAL ACTION OF HUMAN SERUM ON HEMOLYTIC STREPTOCOCCI

I. OBSERVATIONS MADE WITH SERUM FROM PATIENTS WITH ACUTE INFECTIONS AND FROM NORMAL INDIVIDUALS

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The results presented in this communication concern the destructive action upon hemolytic streptococci of serum from patients who are acutely ill. Successive samples of serum have been obtained from representatives of a number of different kinds of infectious diseases, both during acute illness and following recovery, and determinations of bactericidal power have been made by the use of different strains of *Streptococcus hemolyticus* of the *beta* type. The several specimens of sera have exhibited marked differences in streptococcal potency which may be correlated with the condition of the patient at the time at which the blood was obtained. The results which are described in detail in this article were obtained with sera from twenty-five adult patients acutely ill with different infections. The list is as follows:

13 cases of pneumococcus pneumonia.

3 cases of acute respiratory infection of uncertain etiology (lung abscess, bronchitis?, pyopneumothorax).

1 case of tuberculous pleurisy with effusion.

2 cases of acute tonsillitis due to hemolytic streptococcus.

2 cases of meningococcus meningitis.

1 case of staphylococcus septicemia.

3 cases of induced malaria.

Total 25

The attempt was made to select individuals whose illness was not mild but might be expected to be reasonably short and thus afford the

opportunity of making observations as rapid changes in the severity of the condition occurred. The patients with pneumonia or induced malaria were particularly favorable since recovery was usually abrupt and complete. All of the patients were severely or moderately severely ill and in most instances the temperature was high during the acute period. Data with respect to fever and other points are presented in Tables I and II.

Comparable tests were also made with sera from normal individuals; twenty healthy adults, in the third to fifth decades of life, were used for this purpose.

Studies directed toward an understanding of the nature of the reaction have also been in progress and the information, which has accrued from this phase of the investigation, will be given in a second article (1).

Materials and Methods

Serum.—Blood was obtained under sterile precautions. The serum was separated from the clot, usually within a few hours after coagulation had occurred. The specimens were always centrifuged twice in order to insure the removal of leucocytes, and then kept in the ice box until used. Tests were usually carried out on the day after serum was obtained.

Cultures.—18 hour cultures, freshly grown, were always employed. The culture medium consisted of plain meat infusion broth, containing 1 per cent neopeptone and 0.05 per cent dextrose. The pH was adjusted to 7.6, but no buffer was added.

Observations made with many different strains of hemolytic streptococci have revealed differences in susceptibility of the cultures to the bactericidal action of the test sera. Data concerning other characteristics of a large number of strains in relation to sensitiveness to streptococidal action of sera, will be subsequently reported. In the phase of the subject dealt with in this paper, three selected strains were employed. They consisted of the most susceptible strain, the most resistant, and a third occupying an intermediate position.

1. *Strain Sc.*—Most sensitive strain. It was isolated Dec. 9, 1935, from the subcutaneous abscess of a 75 year old patient who died a few days later.

2. *Strain Ba.*—Most resistant strain. It was obtained Mar. 3, 1936, from the finger of a patient with erysipelas.

3. *Strain Co.*—Intermediate strain. It is an old laboratory strain which was recovered Mar. 4, 1931, from the blood of a patient with septicemia.

Before use, the relative abundance of growth of the three cultures was estimated on the basis of the turbidity of the broth. If—as rarely occurred—any difference was noted, the three cultures were reduced to the same degree of density by diluting with sterile broth those which were more turbid.

Test.—1.0 cc. of serum in Wassermann tubes was inoculated with 1 platinum loopful of culture. The loop is 0.2 cm. in diameter. A full, rounded drop of culture for inoculum was obtained by flipping the loop from the liquid while the culture tube was held on a slant. By serial dilutions and subculture in poured plates of blood agar, several observations were made on the number of viable organisms in an inoculum of this size. The estimates ranged from 800,000 to 1,500,000, averaging approximately 1,000,000. After inoculation, the tubes were placed in a water bath at 37.5°C.

Subcultures were made as follows: (a) Serum before introducing organisms. These cultures were always sterile. (b) Serum-streptococcus mixture immediately after inoculation. 1000S to ∞ number of colonies were present. (c) 6 hours after inoculation. (d) 24 hours after inoculation. (e) Occasionally 48 hours after inoculation. For subculture, a loopful of serum comparable in size to that used for inoculum of culture, was put into 10 cc. of melted agar containing 0.7 cc. of defibrinated rabbit blood. The tube was rapidly and vigorously rolled between the palms of the hands and plates were poured. The even distribution of colonies, after growth had taken place, indicated that the procedure afforded satisfactory mixing of serum in the melted agar.

The plates were inspected after 24 hours incubation. If less than 1000 colonies were present, they were incubated for another 24 hours before final reading. The number of colonies in the plates was used as an index of survival or death of streptococci. No attempt was made to differentiate between survival and additional multiplication. The grading of the number of organisms in the plates was as follows:

∞ designates innumerable colonies after 24 hours incubation, with complete hemolysis of the whole plate.

1000S designates a number less than ∞ , but so great that accurate counting was impractical. Small areas of unchanged blood were present in the plates after 24 hours incubation.

When colonies were less numerous, the plate was divided into fourths or eighths and the total was estimated in round numbers from the average of two or three sections. When the number was below 100, all the colonies were counted.

The procedure which has just been described has been used, with various modifications, by numerous investigators. The two objections most frequently advanced against it as a measure of bactericidal action are based on: first, variations in the number of colonies in subculture due to differences in length of chain formation of streptococci, and second, the effect of agglutination by sera on the dispersion of organisms throughout the subculture. In the present experiments, the first of the criticisms is rendered invalid, since repeated microscopical examinations of the serum-streptococcus mixtures demonstrated that there was no correlation between chain formation and

number of colonies. Special consideration has been given to the question of agglutination. Experiments bearing on this point will be described in the second article (1). At the present time it is sufficient to state that many of the sera have been tested with heat-killed organisms at 56°C., and that no antistreptococcus agglutinins have been found.

Before proceeding with a description of the results obtained with sera from patients, data derived from tests made with normal sera will be given. Twenty different adults (sixteen males and four females) supplied specimens of blood. Each of the sera was tested with each of the three strains of hemolytic streptococcus. Repeated specimens were obtained from some of the normal individuals at intervals of several days and weeks. In view of the fact that the results were essentially the same in every instance, they are summarized as follows:

Number of colonies in subculture at beginning of test	:	1000S or ∞
" " " " " after 6 hrs. incubation:	:	∞
" " " " " " 24 " "	:	∞ or 1000S
" " " " " " 48 " "	:	50 to 1000S

As the summary indicates, streptococci maintain approximately the same, or perhaps an increased, population during 24 hours incubation in normal human serum. With the experimental procedures which were used, such slight differences in the three strains as occurred were considered to be negligible. After incubation had lasted for 48 hours, however, there was a definite, but very variable decrease in the number of colonies on subculture of each of the strains. The reason for the late effect is not yet understood. It may represent a delayed and slowly acting process comparable to that exhibited by patients' sera, or it may be dependent upon the influence of extraneous factors such as partial evaporation of water from serum or the accumulation of metabolic products. Since the action of patients' sera occurred in the first 24 hours of incubation and since the late subculture contributed no additional information to the effect of patients' sera, only the observations of the first 24 hours have been analyzed. An explanation of the late effect of normal serum must await further investigation. For the purposes of the experiments with which this study is concerned, the significant finding is the absence of streptococcal action by normal serum during the first 6 to 24 hours of incubation. In every test in which patients' sera were used, a similar test was carried out with normal serum, thus insuring an adequate control.

TABLE I

*Streptococcal Action of Serum from Patients with Lobar Pneumonia (Pneumococcus)
Tested with Three Strains (Sc, Co, and Ba) of Hemolytic Streptococci*

The number of colonies present in subcultures made at beginning of experiment was always 1000S to ∞

Patient	Clinical course	Day of disease	Temperature	Sc*		Co*		Ba*	
				6 hrs.†	24 hrs.†	6 hrs.	24 hrs.	6 hrs.	24 hrs.
M. F.	Recovery 9th-10th day. Pneumococcus group IV	4	103.6	—†	—	20	1	∞	1000S
		7	103.6	2	—	2	—	∞	∞
		15	99.4	∞	1000S	∞	∞	∞	1000S
		23	99.8	∞	∞	∞	∞	∞	∞
W. S.	Recovery 10th-11th day. Pneumococcus Type X	5	104.6	—	—	2	—	1000	1
		13	99.4	150	—	∞	∞	∞	∞
		20	99.0	∞	1000S	∞	∞	∞	∞
G. J.	Marked improvement 10th day. Low fever until discharge 34th day	6	101.2	—	—	350	—	∞	1000S
		13	100.0	∞	1000S	∞	∞	∞	1000S
R. C.	Rapid recovery 2nd-3rd day. Pneumococcus group IV	1-2	102.8	—	—	4	—		
		5	98.6	∞	∞	∞	1000S	∞	∞
W. T.	Crisis 9th day. Pneumococcus group IV	3	104.0	15	—	22	4	1000S	800
		7	105.4	90	—	150	—		
		14	98.8	∞	∞	∞	∞	∞	∞
C. S.	Gradual improvement beginning 9th day. Febrile until 13th day. Pneumococcus Type III	2	105.2	3	—	—	—	1000S	200
		10	101.8	2	—	—	—	∞	∞
		15	99.4	22	—	∞	∞	∞	∞
		20	99.2	240	2	∞	2		
		28	98.6	∞	∞	∞	∞		
O. T.	Bronchopneumonia with low grade fever until 23rd day	7	101.6	1	—	1000S	—	∞	350
		28	99.8	∞	1000S	∞	∞	∞	∞

* Designates strain employed in experiments.

† Hours indicate length of incubation before subculture was made.

‡ The sign (—) indicates no growth in subculture. The numerals indicate number of colonies in subcultures. 1000S and ∞ are approximate estimations when actual counts were impractical.

TABLE I—*Concluded*

Patient	Clinical course	Day of dis- ease	Tem- pera- ture	Sc*		Co*		Ba*	
				6 hrs.†	24 hrs.†	6 hrs.	24 hrs.	6 hrs.	24 hrs.
F. R.	Pneumococcus Type I. Serum-treated. Marked improve- ment 4th day. Low fever until 14th day	3	104.0	1	—	4	—	1000S	55
		5	99.0	5	—	28	—	∞	1000S
		15	99.2	∞	6	∞	1000S	∞	∞
		22	99.0	∞	∞	∞	∞	∞	∞
J. M.	Pneumococcus Type I. Serum-treated. Marked improve- ment 6th day. Low fever until 12th day	4	104.0	1	—	114	3	1000S	1000S
		8	101.0	71	9	∞	∞	∞	∞
		13	99.8	∞	∞	∞	1000S	∞	1000S
A. F.	Pneumococcus Type II. Serum-treated. Definite improvement 4th day	3	102.4	2	—	350	18	1000S	∞
		7	99.6	∞	15	∞	∞		
		12	99.4	1000S	2				
		15	99.6	∞	2	∞	∞	∞	∞
J. A.	Pneumococcus Type I. Serum-treated. Operation for em- pyema 36th day	4	104.0	—	—	—	—	1000S	1000S
		13	101.8	400	—		1		
		21	101.2	65	—	175	11		
		39	100.0	∞	5	∞	∞		
		47	99.4	∞	∞	∞	∞		
E. P.	Pneumonia, septi- cemia, endocar- ditis. Pneumococ- cus Type X. Died	11?	104.8	—	—			280	—
		16?	102.5	—	—			80	—
		18?	102.4	—	—	4	—	400	—
		24?	103.5	2	—	2	—	250	2
		33?	103.0	—	—	3	—	300	—
L. B.	Pneumococcus group IV. Serum ob- tained 6 hrs. before death	7	103.2	4	—	325	12		250
20 nor- mal adults				∞	∞ to 1000S	∞	∞ to 1000S	∞	∞ to 1000S

The greatest number of observations were made with serum from patients having pneumococcus pneumonia. The results are presented in Table I. The table has been so arranged that, in the section assigned to each patient, a space separates, where possible, the period of acute illness from that of recovery or convalescence. The differences in the data recorded above and below this space reveal the changes in streptococcal action of sera from each patient for each of the strains.

From Table I it may be seen that all of the sera taken during the acute stage of the illness were capable of destroying organisms of strain Sc, the most sensitive of the three. The number of colonies in subcultures made after 6 hours incubation decreased rapidly from the thousands present at the beginning of the experiment to less than fifteen or even none. The 24 hour subcultures were regularly sterile. With ten of the specimens, the whole cubic centimeter of serum was subcultured after 24 hours. No organisms were recovered in eight instances, and less than ten colonies grew from subcultures of the remaining two specimens. The complete destruction of all the bacterial cells is additional evidence that the phenomenon is a true bactericidal one.

The bactericidal action of these same sera on strain Co is also definite but, in some instances, proceeded at a slightly slower rate than on strain Sc. When strain Ba was used, evidence of the bactericidal action was obtained with the sera of only six of the thirteen patients; the remaining seven were as ineffective as normal sera against this strain. The difference in susceptibility of the three strains is also brought out by the results presented in Table II.

It may be seen, therefore, that lobar pneumonia is attended by the presence of a serological property which is antagonistic to the survival of an organism of a different species, and that the streptococcal element was demonstrable in serum obtained within 48 hours of the beginning of pneumonia—patients R. C. and C. S., Table I—and persisted so long as the infectious process was active.

It may be further noted from the table that the effectiveness of the early samples of sera is no less striking than the absence or diminution of streptococcal activity in specimens derived from patients after they had recovered. Within a week following the patient's critical recovery or marked improvement, the bactericidal property

TABLE II

Streptococidal Action of Serum from Patients with Different Infections Tested with Three Strains (Sc, Co, and Ba) of Hemolytic Streptococci

The number of colonies present in subcultures made at beginning of experiment was always 1000S to ∞

Patient	Bacteriology	Clinical course	Day of disease	Temperature °F.	Sc		Co		Ba	
					6 hrs.	24 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.
R. B.	<i>Streptococcus hemolyticus</i>	Acute tonsillitis. Temperature normal 10th day. Rapid recovery	8	103.4	1	—	105	—	∞	∞
			13	98.6	∞	1000S	∞	∞	∞	∞
G. C.	<i>Streptococcus hemolyticus</i>	Peritonsillar abscess, cervical adenitis. Marked improvement 6th day. Low fever 15 days	2	104.0	—	—	—	—	750	19
			6	102.8	16	—	78	—	1000S	1000S
			20	99.2	∞	1000S	∞	∞	∞	1000S
R. J.	Meningococcus	Meningitis. Serum-treated. Marked improvement 5th day	3	101.5	18	2	220	—	∞	∞
			7	100.0	165	90	∞	—	∞	∞
			14	98.4	∞	∞	∞	1000S	∞	∞
W. J.	Meningococcus	Meningitis. Serum-treated. Died 5th day	2		—	—	18	2		
G. J.	<i>Staphylococcus aureus</i>	Multiple abscesses. Septicemia. Died 25th day	10	102.2	42	2	200	—	∞	∞
			17	103.8	7	—				
A. M.	<i>Bacillus tuberculosis</i>	Pleurisy with effusion. Progressive gradual improvement	8?	102.0	83	—	∞	2	∞	500
			15?	101.4	1000S	—	800	32	∞	320
			25?	100.2	210	1	∞	∞	∞	∞
			39?	100.0	∞	92	∞	∞	∞	∞

T. D.	Uncertain	Acute respiratory infection. High fever 4 days. Marked improvement 13th day	8 17 22	104.0 98.6 98.6	— 600 ∞	— 3 1000S	— 250 ∞	— ∞ ∞	∞ ∞ ∞	1000S ∞ ∞
D. K.	<i>H. influenzae</i> <i>B. coli</i>	Pyopneumothorax. Fever for 7 wks. Operation	567 867	102.2 99.6	82 ∞	— ∞	— ∞	— ∞	∞ ∞	∞ ∞
G. A.	Uncertain	Lung abscess? Progressive gradual improvement	237 387 457	103.0 99.5 99.8	46 ∞ ∞	6 5 180	220 ∞ ∞	56 ∞ ∞	∞ ∞ ∞	1000S 98 1000S ∞
H. S.	Induced malaria	1st serum before infection. 2nd serum on 3rd day of fever, during chill. 3rd serum be- tween chills. Last day of fever 19th	4 11 16 21 29	99.0 104.0 99.4 105.0 98.2 98.8	∞ 54 140 32 ∞ ∞	1000S — — — 6 ∞	∞ 1000 ∞ 1000S ∞	1000S — 3 150 1000S	∞ ∞ ∞ ∞ ∞	1000S 98 1000S ∞
W. F.	Induced malaria	Last day of fever 20th	14 19	106.2 103.2	19 1000	— 4	∞ ∞	— ∞	∞ ∞	160 1000S
C. T.	Induced malaria	Last day of fever 35th	23 16 24 32 40	99.0 101.2 105.8 105.2 99.0	∞ 240 52 170 ∞	1000S 3 — 1 ∞	∞ ∞ ∞ ∞ ∞	∞ — 2 ∞	∞ ∞ ∞ ∞	1000S ∞
20 normal adults					∞	∞ to 1000S	∞	∞ to 1000S	∞	∞ to 1000S

For an explanation of symbols, see Table I.

for streptococci was either lost or definitely reduced in potency. In every instance but one, the last specimen of serum, obtained before discharge of the patients from the hospital, was indistinguishable from normal serum. One patient (J. A.) developed empyema. Repeated tests with his sera showed that the killing power persisted until after successful operative treatment.

Two patients, represented at the bottom of the table, died. Patient E. P. had pneumonia with septicemia and then developed bacterial (pneumococcus) endocarditis. The number of colonies of pneumococci in her blood cultures varied irregularly during the infection from none to several hundred. She died after an illness of 6 weeks. Her serum was continually one of the most potent with which these observations have been made. Patient L. B. had pneumonia with septicemia and died 7 days after onset. With the sample of her blood, obtained 6 hours before death, streptococcal action was present to a high degree.

The results presented in Table II were obtained from similar tests made with sera from patients having acute diseases of different etiologies. Examples of infection with hemolytic streptococcus, meningococcus, staphylococcus, tubercle bacillus, and malarial parasites are presented. Although the number of individuals representative of each type of infection is few, the results are in every way comparable to those obtained with cases of lobar pneumonia due to pneumococcus. The streptococcal property was high in specimens of serum taken during active disease and either disappeared within a few days after rapid recovery, or gradually diminished, when improvement was slow, as in the case of tuberculous pleurisy with effusion and that of pulmonary abscess.

From the data presented in Tables I and II, it may be concluded that the human serological property responsible for the destruction of hemolytic streptococci is evoked by different disease-producing agents and that its presence, which is transient, is determined by the activity of the infection. The difference in the rate and completeness of the destruction of the three strains is also noteworthy.

In reviewing the very extensive literature¹ on the subject of the bactericidal action of blood, a few articles have been found which contain results somewhat

¹ Some idea of the extent of the literature concerning the bactericidal action of blood may be gained by referring to the article by Knorr (4); the text is followed by 24 pages of references with approximately 30 titles per page. The army of

comparable to those just described. Dresel and Keller (2) reported results of observations on bactericidal action of serum from patients on anthrax bacillus (*Anthrakocidin*). They found that the serum from normal persons did not possess the property, but that it was present in serum from patients having a wide variety of acute or chronic diseases of an infectious or degenerative nature, such as tuberculosis, syphilis febrile or afebrile, pernicious anemia, leucemia, cirrhosis of liver, cardiac disease, and arterial sclerosis. They also noted that this property of the serum may be present for a considerable length of time after recovery or improvement. Trommsdorff (3) tested the killing power of serum from patients for staphylococci, typhoid and colon bacilli, and obtained evidence of bactericidal action. The latter two organisms were more easily destroyed than staphylococci. He noted that five normal sera were somewhat bacteriostatic but not bactericidal, and that among the sera from nine patients, six of whom had staphylococcus septicemia, five were listed as strong or moderately strong in bactericidin and four as weak or minimal. He tested further for alexin by determining the hemolysin titer of the sera and found some degree of parallelism between the two tests. He comments upon the fact that the alexin content of sera from both normal persons and patients may be variable in successive samples, and concludes that the quantitative determination of its presence in human sera is neither diagnostic nor prognostic.

The fact that extracts of leucocytes (leukin) have been found to be bactericidal has led some investigators to offer the suggestion that the bactericidin of serum may be derived from leucocytes. Among the patients included in this present study leucocytosis has often been present when the serum was highly bactericidal for streptococci. There were several exceptions, however, in cases with normal or leucopenic white blood cell count, but with sera markedly effective in streptococidal activity. Consequently, a superficial correlation between number of leucocytes in the circulating blood and bactericidal potency of the serum offers no information concerning the suggested leucocytic origin of the streptococidal properties.

All of the patients had fever, often 103°F. or higher, and often con-

investigators in this field have used whole blood or defibrinated blood or serum or leucocytes. Blood has been obtained from many different animal species both normal and immunized. A wide variety of bacterial species have been employed. It is interesting to note that Topley and Wilson (5) state that "There are not many bacteriological problems which present quite so confusing and unsatisfactory a collection of data as do those concerned with the nature and mechanism of the bacteriolytic and bactericidal antibodies." In this article and the one that follows, citation of references has been limited to a few which seem to be directly pertinent to the subject matter under consideration.

tinuous, during the acute period of sickness. Furthermore, the permanent disappearance of fever was closely followed by the loss of destructive action of serum. Accurate information concerning the significance of fever has not yet been obtained. However, patients receiving malaria by inoculation have afforded the opportunity of making observations which may be applicable to this aspect of the problem. Of the individuals with general paresis, who were admitted to the hospital for malarial therapy, data derived from three are given in the lower part of Table II and are representative of similar results, obtained with blood from the others. All the data obtained with sera from patients with malaria may be summarized as follows: From three of the patients, serum was gotten before treatment was begun. The outcome of the tests was similar to that obtained with normal serum, indicating that the chronic syphilitic infection did not evoke the serological response of acute infections. From two of the patients, the second sample of blood was acquired within 24 to 48 hours of the first rise in temperature. Streptococcal action was demonstrable with both of the specimens. Subsequent bleedings were made both at times of highest fever and during temporary remissions of 1 to 3 days. Under both conditions, the capacity to destroy streptococci was present in the sera, but the technique which was used did not reveal any consistent quantitative differences. However, when the infection was terminated by quinine, within the following 2 to 3 days the activity of the specimens of the patients' sera returned to the normal level of the pre-infection sample. In these few observations, therefore, it appears that the streptococcal element is maintained in the blood of patients with malaria regardless of the time in the swings of fever at which the blood is taken. With the permanent loss of fever, however, the blood rapidly returns to normal.

A comparison of the results obtained with sera from cases of pneumonia with that derived from cases of malaria suggests that the former specimens were more potent in streptococcal power. This is of interest in view of the fact that the duration of active infection in pneumonia was often not as prolonged as in malaria and also that the fever in pneumonia, although usually continuous instead of intermittent, did not regularly reach the height exhibited by the malarial patients.

Although the methods which were employed afforded only a rough quantitative measure of streptococidal activity, the impression has been gained that sera of greatest killing power came from individuals whose illness was most severe.

DISCUSSION

The observations which have been recorded demonstrate the striking capacity of sera from patients with acute infections to destroy hemolytic streptococci. Under the experimental conditions employed, the rapidity with which the number of viable organisms introduced into the serum was found to diminish and, finally, to disappear completely, is indicative of the potency of the serological property responsible for the bactericidal effect. By making repeated observations on successive samples of serum from the same individual it was found that recovery of the patient was followed by a great decrease in killing action of the blood serum. With blood from patients whose recovery was abrupt, as in pneumonia terminating by crisis or in malaria responsive to quinine, the rapid loss of the streptococidal power of the serum following the cessation of active disease was very striking. When improvement was slower, the decrease in the serological property was also delayed. The results indicate that when the stimulus of active infection was overcome, the body factors, whatever their nature, which are responsible for the continued presence in the blood of the bactericidal property, also ceased to operate.

From the tables it may be noted that there is a relatively close parallelism between the bactericidal capacity of the serum and the temperature of the patient at the time blood was withdrawn. It will be of interest to attempt to determine whether fever itself is of prime importance in influencing changes in the blood serum or whether other factors inherent in active febrile disease cause the appearance and disappearance of the humoral bactericidal property.

The time relationship between the presence or absence of the streptococidal property and the course of the disease, which has just been discussed, exemplifies a difference between this particular antibacterial activity and the well defined antibodies reactive with bacteria such as agglutinins. Furthermore, the fact that sera from patients having pneumococcus, hemolytic streptococcus, staphylo-

coccus, meningococcus, or malarial infections, all possess the streptococidal property, is an additional observation which differentiates this reaction from the specific antibacterial response which so commonly follows infections.

The differences in the susceptibility of the three strains of hemolytic streptococci which were employed in this study, are also of interest. One strain (Sc) was destroyed by sera from all of the acutely ill patients. On the other hand, the viability of another strain (Ba) was affected by only a few sera. The third strain (Co) possessed a degree of sensitiveness intermediate between the other two. The results obtained with the three separate strains were used as a rough measure of the streptococidal potency of the sera. Specimens able to act upon all three strains were considered to be the most powerful; those affecting strains Co and Sc were deemed to be somewhat less so; and finally, the sera capable of killing only strain Sc were classified as the least potent. Proof of the correctness of this assumption must await further investigation with a technique which will afford more accurate quantitative measurements. However, it should be emphasized that differences in the behavior of strains need to be taken into consideration in performing experiments such as those which have been described.

SUMMARY

Sera obtained from patients at the time of acute active infections were found, in every instance, to be bactericidal for hemolytic streptococci. The observations were made with sera from twenty-five patients. The group consisted of cases of pneumococcus, hemolytic streptococcus, staphylococcus, meningococcus, tubercle bacillus, and malarial infections; the etiology of the diseases in other patients was either uncertain or may have been a mixed infection.

In sera taken from the same group of patients, soon after recovery or marked improvement had taken place, the streptococidal property was absent or greatly diminished in potency.

By comparative tests made with sera from twenty healthy adults, the streptococidal action was not demonstrable.

Three different strains of *Streptococcus hemolyticus* of the beta type

were employed in the experiments. Differences in susceptibility of the strains to the killing power of the serum were noted.

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THE BACTERICIDAL ACTION OF HUMAN SERUM ON HEMOLYTIC STREPTOCOCCI

II. FACTORS WHICH INFLUENCE THE PHENOMENON IN VITRO

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The observations described in the preceding article (1) have demonstrated that sera, obtained from patients during acute active infection, are lethal for *Streptococcus hemolyticus* of the beta type. In addition, it was shown by contrast that subsequent samples of serum taken from the same group of patients very soon after they had recovered, and that specimens from normal adults, were essentially devoid of the streptococidal property, when measured by comparable tests. Consistent results were obtained by an experimental method which took into consideration the following factors: (a) the amount of serum used for the test; (b) number of bacterial cells inoculated into serum; (c) selection of test strains of hemolytic streptococci; (d) periods of exposure of organisms to the poisoning effect of serum. The results indicate that the streptococidal property of serum is demonstrable early in acute infection, that it is maintained during the period of active disease, but returns to the normal level very soon after the illness has been overcome.

The investigation has been extended in an attempt to gain some information concerning the processes involved in the serological phenomenon. It is the purpose of this article to describe certain conditions which play an important rôle in determining the results of tests, *in vitro*, and to indicate the possible application of the findings to an interpretation of the underlying mechanism. Additional observations, which aid in the characterization of streptococidal sera, are also contained in this report.

The same experimental technique which was described in detail in the preceding article (1) has been used throughout the investigation.

Effect of Anaerobic Conditions on the Streptococcal Action of Serum

In addition to the routine method of carrying out the experiments, sera, containing the regular inoculum of culture, have been incubated in the partial anaerobic state afforded by a vaseline seal and also under the more completely anaerobic conditions of an anaerobic jar.

1. Vaseline Seal.—Technique: 1.0 cc. of serum was inoculated with a platinum loopful of culture, as in the usual test (1). Strain Sc was employed. The tubes were shaken in order to mix the contents well, but care was taken to avoid the formation of foam. 1.0 to 1.5 cc. of sterile melted vaseline was layered on top of the serum in a Wassermann tube as soon as possible after the organisms were introduced.

Because of the simplicity of the procedure, almost every sample of serum from patients was tested both with and without a vaseline seal. Representative results from a large number of tests are given in Table I. The data selected for tabulation were obtained, in most instances, with portions of the same sera used in the previous study (1). To facilitate comparisons, Table I of this article follows, with respect to the listing of patients, the order of Tables I and II of the preceding article.

Observations have been repeatedly made with normal serum. Since colonies, too numerous to count, were obtained in both aerobic and anaerobic tests, no attempt was made to compare the relative abundance of organisms in normal serum under the two conditions.

The thirty observations recorded in Table I, were made with sera from twenty different patients, twelve of whom had pneumococcus pneumonia and the remaining eight had different kinds of infections. They were all acutely ill at the time at which the blood was taken. The contrast between the sterility, on the one hand, of subcultures made after 24 hours of aerobic incubation of the tests, and the abundance of streptococci, on the other hand, in subcultures of the sealed specimens, is the striking feature of the results. With twenty-four of the thirty specimens of serum, the profound influence of anaerobiosis on the phenomenon is evidenced by the difference between the figures in the column designated aerobic, 24 hours and the indices of bacterial

survival in the column which is headed vaseline seal, 24 hours. Under the conditions of the experiment, the death or survival of the organisms in potentially streptococcal sera appeared to be determined, to a

TABLE I

Effect of Anaerobic Conditions (Vaseline Seal) on Streptococcal Tests with Patients' Sera

Number of colonies present in subcultures made at beginning of experiments was always 1000S to ∞

Patient	Disease	Sample* of serum	Aerobic			Patient	Disease	Sample of serum	Aerobic		
			6 hrs.†	24 hrs.†	Vaseline seal 24 hrs.†				6 hrs.	24 hrs.	Vaseline seal 24 hrs.
M. F.	Pneumonia	1	—	—	8	A. F.	Pneumonia	1	2	—	8
W. S.	"	1	—	—	—	E. P.	"	2	—	—	8
"	"	2	150	—	800	"	"	3	—	—	34
G. J.	"	1	—	—	8	"	"	4	2	—	8
R. C.	"	1	—	—	8	"	"	5	—	—	7
C. S.	"	1	3	—	10	L. B.	"	1	4	—	8
"	"	2	2	—	500	R. B.	Acute tonsillitis	1	1	—	1000
"	"	3	22	—	∞	G. C.	Peritonsillar abscess	1	—	—	1000S
O. T.	"	1	1	—	8	"	"	2	16	—	8
F. R.	"	1	1	—	24	R. J.	Meningitis	1	18	2	8
"	"	2	—	6	8	A. M.	Pleurisy	1	83	—	1000S
J. M.	"	1	1	—	8	T. D.	Acute respiratory infection	1	—	—	8
"	"	2	71	9	∞	H. S.	Malaria	2	54	—	8
J. A.	"	1	1	—	62	W. F.	"	1	19	—	8
"	"	2	400	—	∞	C. T.	"	2	52	—	8

* Identifies sample of serum from individual patients. 1 for first, 2 for second, etc.

† Indicates number of hours of incubation before subculture was made.

‡ Sign — indicates sterile subculture. Numerals indicate number of colonies in subcultures. 1000S and ∞ are approximate estimations when actual counts were impractical.

large extent, by factors which were effective under conditions of oxidation but were inactive under circumstances which induced reduction.

It should be noted also that, with six of the specimens of serum,

exclusion of air modified the killing action only slightly, or not at all. Final explanation of the exceptions is not yet available. However, a probable interpretation is suggested by the outcome of some of the experiments which immediately follow.

2. *Anaerobic Jar*.—In order to obtain more complete anaerobiosis than that afforded by a layer of vaseline, additional tests have been carried out in a Brown anaerobic jar (2). In this apparatus, which employs the same principle as

TABLE II

Comparison of Streptococcal Action of Patients' Sera under Aerobic Conditions, with Vaseline Seal, and in Anaerobic Jar

Number of colonies present in subculture at beginning of experiment was always 1000S to ∞

Patient	Disease	Aerobic				Vaseline seal		Anaerobic jar	
		Sc*		Co*		Sc	Co	Sc	Co
		6 hrs.†	24 hrs.†	6 hrs.	24 hrs.	24 hrs.	24 hrs.	24 hrs.	24 hrs.
B.	Pneumonia	125†	4			∞		∞	
M.	"	1	—	114	3	∞	∞	∞	∞
C.	"	25	2	350	6	∞	∞	∞	∞
T.	Malaria	160	—			∞		∞	
S.	"	54	—	1000	—	∞	∞	∞	∞
C.	"	125	3	800	15	∞	∞	∞	∞
F.	"	121	—			∞		∞	
V.	"	68	—			∞		∞	
H.	"	142	—			∞		∞	
H.	Lung abscess	142	—			∞		∞	
W.	Pneumonia	—	—	26	1	10	1000S	41	1000S
S.	"	2	—	120	—	86	400	—	1000S
R.	"	1	—	4	—	24	∞	94	∞
C.	Tuberculosis	72	—	210	—	—	1000	—	780

* Designates strain of hemolytic streptococcus used in tests.

†, ‡ See Table I.

the McIntosh and Fildes jar, oxygen is exhausted in the formation of water and an excess of hydrogen remains. The sera to be tested were placed in the jar immediately after inoculation with culture. A tube of sterile dextrose broth, containing methylene blue, was added as an indicator of anaerobiosis. During the 24 hours of incubation the methylene blue regularly changed to methylene white.

The findings recorded in Table II afford a comparison of the streptococcal effect of sera under aerobic conditions, under partial anaerobic state obtained with a vaseline seal, and in an anaerobic jar.

From the table it may be noted that, with nine specimens of sera—listed in order at the top of Table II—streptococci survived and perhaps multiplied, when either of the two anaerobic methods was employed in the tests. By contrast, progressive bacterial destruction occurred aerobically. A comparison of the results obtained with the anaerobic jar and vaseline seal demonstrate the inactivation of the lethal process by both procedures. However, the observations do not indicate the superiority of one method over the other in accomplishing the particular purpose for which the experiment was designed. For example, in four tests listed at the bottom of Table II, strain Sc was destroyed almost to the same extent anaerobically as aerobically; even the complete anaerobiosis of the jar was not more effective than the vaseline seal. However, by substituting the somewhat less sensitive strain Co for strain Sc and repeating the test with the same four specimens of sera, the inactivating effect of anaerobiosis on the streptococcal process became evident. The results of these observations indicate, therefore, that the outcome of some of the experiments may be conditioned by a balance between the potency of the serum in streptococcal activity and the vulnerability of the test strain.

In connection with the observations just described, it is interesting to call attention to an article by Schottmüller and Barfurth (3). They reported results concerning the bactericidal action of defibrinated blood on streptococci of the hemolytic, anhemolytic, and *viridans* types. They asserted that the bactericidin in *sauerstoffhaltigen* blood was greater than in *kohlensäuredurchhaltigen* blood, and that factors, such as increased water content of the blood or decreased number and resistance of erythrocytes, lessened the bactericidal power of the blood.

Effect of Reversing the Aerobic-Anaerobic System

Tests have been performed in which the aerobic and anaerobic conditions were altered during incubation of sera inoculated with streptococci. The purpose of the experiments was to determine: (a) whether the streptococcal process could be interrupted by anaerobiosis if tests were allowed to proceed aerobically for varying lengths of time before excluding air; and (b) whether the destructive action was permanently inactivated by short periods of anaerobiosis before exposure to air.

The method consisted in placing 1.0 cc. of serum into a series of tubes and inoculating each with a loopful of culture. Strain Sc was used in all the experi-

ments. Subcultures from each tube were made immediately; then, half of the series was layered with vaseline. All of the tubes were incubated continuously for 24 hours; at the end of this time a final subculture was made and the experiment was terminated. At selected intervals, ranging from 2 to 12 hours, vaseline was added in succession to each of the aerobic tubes; subcultures were made just before sealing. From each tube of the anaerobic set, vaseline was carefully removed in series, after periods ranging from 2 to 12 hours; subcultures were made promptly and, then, incubation was continued aerobically until the termination of the experiment. Additional subcultures from the tubes incubating aerobically could be made as desired. When anaerobiosis was continuous, the interruption of the tests to obtain subcultures necessitated the removal and replacement of a vaseline seal. When this procedure was used, it was carried out as rapidly as possible, in order to minimize the period of transient exposure to air. The unavoidable error in technique has not significantly affected the results.

Table III contains four examples of the effect which reversing the aerobic or anaerobic status of individual tests had upon the survival or destruction of hemolytic streptococci in single samples of serum.

From the data derived from tests made with sera from three of the patients (V., L., and C.), it may be noted that when a vaseline seal was added to an aerobic test within 2 hours after the serum had been inoculated with organisms (tubes 2, Table III), the destruction of streptococci was checked, as evidenced by the innumerable colonies present in subcultures made after the subsequent 22 hours of anaerobic cultivation. When aerobic conditions prevailed for 4 hours before adding a layer of vaseline (tubes 3, Table III), the surviving streptococci in one specimen of serum (patient L.) multiplied under anaerobiosis, but with the other two sera, similarly tested, the number of viable organisms recovered after the subsequent 20 hours of anaerobic incubation was not significantly increased over the few present when air was first excluded. By prolonging the aerobic phase of the experiment for 6 hours before excluding air (tubes 4, Table III), the lethal process was inactivated very slightly, if at all, by adding a vaseline seal. Finally, when aerobic incubation proceeded for 12 hours, the subsequent exclusion of air did not alter the final result.

The tabulated results derived from the tests made with serum from patient B. indicate that the interchange of aerobic and anaerobic conditions modified the outcome only to a limited extent. Comparable results have been obtained with some other specimens of sera.

TABLE III

Reversibility of Effect of Aerobic and Anaerobic Conditions on Streptococcidal Reaction of Patients' Sera

Number of colonies present in subculture at beginning of experiment was always 1000S to ∞

1000S to 1000S

Tube No.	Aerobic and anaerobic conditions	Duration of incubation before subculture					Tube No.	Duration of incubation before subculture					
		2 hrs.†	4 hrs.	6 hrs.	12 hrs.	24 hrs.		2 hrs.	4 hrs.	6 hrs.	12 hrs.	24 hrs.	
Patient V. (induced malaria)							Patient L. (broncho-pneumonia)						
1	Aerobic 24 hrs.	600†	140	68	4	—	1	200	41	6		—	
2	Aerobic 2 hrs.	510			650	∞	2	160				1000S	
	Anaerobic 22 hrs.												
3	Aerobic 4 hrs.	550	86		2	61	3		34			∞	
	Anaerobic 20 hrs.												
4	Aerobic 6 hrs.			82	7	22	4			10		27	
	Anaerobic 18 hrs.												
5	Aerobic 12 hrs.			51	4	—	5				—	—	
	Anaerobic 12 hrs.												
6	Anaerobic 24 hrs.	710		1000S	∞	∞	6					∞	
7	Anaerobic 2 hrs.	580	90		3	—	7	240				—	
	Aerobic 22 hrs.												
8	Anaerobic 4 hrs.		960		2	—	8		340			—	
	Aerobic 20 hrs.												
9	Anaerobic 6 hrs.			∞	∞	1000	9			600		6	
	Aerobic 18 hrs.												
10	Anaerobic 12 hrs.			∞	∞	∞	10				1000S	800	
	Aerobic 12 hrs.												
Patient C. (tuberculosis)							Patient B. (lobar pneumonia)						
1	Aerobic 24 hrs.	750	54	6	5	1	1	140	45	5	1	—	
2	Aerobic 2 hrs.	600				1000S	2	104				16	
	Anaerobic 22 hrs.												
3	Aerobic 4 hrs.		115			300	3		16			8	
	Anaerobic 20 hrs.												
4	Aerobic 6 hrs.			11	—	220	4						
	Anaerobic 18 hrs.												
5	Aerobic 12 hrs.				15	18	5				—	—	
	Anaerobic 12 hrs.												
6	Anaerobic 24 hrs.					∞	6					∞	
7	Anaerobic 2 hrs.	∞		1000S		160	7	91		2		—	
	Aerobic 22 hrs.												
8	Anaerobic 4 hrs.		∞			1000	8		47		10	3	
	Aerobic 20 hrs.												
9	Anaerobic 6 hrs.			∞	∞	1000S	9						
	Aerobic 18 hrs.												
10	Anaerobic 12 hrs.				∞	∞	10				200	350	
	Aerobic 12 hrs.												

†, †† See Table I.

In these instances, inactivation of the streptococcal process was completely successful only in the tests maintained in continuous anaerobiosis for 24 hours, although a few organisms ultimately survived short periods of aerobic incubation. Sera, yielding results such as that just described, have been found, by other tests, to possess an unusually high degree of killing power. From a large number of observations a consistent relationship has been noted between the streptococcal potency of individual specimens of serum and the critical period of incubation which determines the reversibility of the reaction. Additional evidence that the quantitative factor is important has been obtained by experiments in which the amount of inoculum of culture introduced into highly potent sera, was increased severalfold. The results, under these circumstances, are comparable to those obtained when the regular amount of culture was added to other sera.

In spite, therefore, of limitations which surround the experimental procedure just described, the results demonstrate that, if the progressive killing of streptococci is not allowed to continue too long, the streptococcal effect, which occurs aerobically, may be altered by introducing a state of anaerobiosis.

In the second part of the experiment, relating to reversibility, the tests were begun under anaerobic conditions and subsequently changed to aerobiosis (tubes 6 to 10, Table III). In three instances, the reactivation of the lethal effect following exposure to air was demonstrable provided the vaseline seal was not allowed to remain in place longer than 6 hours before being permanently removed. When 12 hours were allowed to intervene between the change from anaerobic to aerobic state, the final subculture usually contained large numbers of colonies. (In the last tests in this group, however, (tubes 9, Table III) the period of aerobic incubation may have been so short that the lethal process, if active, was not demonstrable.) As previously stated, the ultimate survival of streptococci in the highly potent serum from one patient (B.) required uninterrupted anaerobic incubation.

It is interesting to call attention to the fact that, in the tests initiated under anaerobiosis, subcultures after 2 to 4 hours incubation often revealed a temporary decrease in bacterial population. That the killing action occurring in these instances was, however, not

sustained, is evidenced by the large numbers of viable organisms which were ultimately demonstrable when anaerobiosis was continued for 24 hours. The result indicates that the lethal action may proceed for a period of time even under anaerobic conditions. The probable explanation of the transient streptococcidal effect occurring anaerobically rests upon a period of lag between the addition of vaseline and the ultimate induction of a sufficient degree of anaerobiosis to inactivate the process. The simple methods, which have been employed in the present study, do not offer the means for a detailed analysis of this phase of the problem. Preliminary trials with chemical reductants have, therefore, been made. The results, although incomplete, suggest that the use of reducing agents or a control of oxidation-reduction potentials by appropriate reagents will afford a more accurate assay of the aerobic-anaerobic conditions involved in the phenomenon. At the present time, however, even with the methods which have been employed, it may be concluded that the anaerobic as well as the aerobic effect on the streptococcidal reaction is, under proper conditions, reversible.

Effect of Heat on the Streptococcidal Action of Patients' Sera

Twelve specimens of sera, each from a different patient, have been heated at 56°C. and 60°C. respectively, for 1 hour. The results are given in Table IV.

From the data it may be seen that the results are not uniform. In nine of the twelve instances, heating at 60°C. either destroyed the lethal property of sera or definitely impaired it. With three specimens, each derived from a patient severely ill with pneumonia, the effect of heating even at 60°C. was not striking. When exposure to 56°C. was used, the streptococcidal action of half the specimens was either completely or partially inactivated, but the remaining six samples retained the capacity to destroy streptococci. Although there were exceptions, heat was less deleterious for sera which were considered to be highly potent in lethal properties than for specimens which were deemed to be less active.

Maintenance of Streptococcidal Action by Sera Kept in the Ice Box

In the former article (1) it was stated that serum was usually tested within 24 hours of the time that it was obtained from the patient. In

order, however, to gain information as to the lability or stability of the streptococcal property, some of the specimens of sera have been retested after being kept in the ice box for as long as 4 weeks. The limited number of observations indicate the destructive action is retained unimpaired for 3 weeks and that there is only slight decrease even after standing for 4 weeks in the ice box.

TABLE IV

Effect of Heat on Streptococcal Property of Patients' Sera

Number of colonies present in subcultures at beginning of experiment was always 1000S to ∞

Patient	Disease	Unheated		Heated 56°C., 1 hr.		Heated 60°C., 1 hr.	
		6 hrs.†	24 hrs.†	6 hrs.	24 hrs.	6 hrs.	24 hrs.
P.	Pneumonia	2‡	—	—	—	1000S	8
T.	"	15	—	—	—	1000S	500
C.	"	—	—	—	—	41	—
M.	"	46	—	∞	∞	∞	∞
S.	"	120	—	∞	1000	∞	∞
L.	"	6	—	48	—	52	—
B.	"	5	—	8	—	48	2
D.	"	100	—	∞	∞	∞	∞
L.S.	"	22	—	65	—	1000	14
S.	Bronchopneumonia	360	—	1000S	8	∞	∞
R.	"	1	—	800	9	∞	1000S
V.	Malaria	6S	—	1000S	800	∞	∞

†, ‡ See Table I.

Consideration of Agglutination

In order to determine whether or not agglutination may be a factor in the bactericidal tests employed in this study, observations have been made concerning both true agglutination and pseudo-agglutination of hemolytic streptococci by patients' sera.

A procedure, commonly used in determining the presence of agglutinins in serum for hemolytic streptococci, consists in adding to appropriate dilutions of serum, organisms which have been killed by heat. The test is then incubated at 56°C. for 2 hours followed by overnight exposure to ice box temperature. Many of the sera used in the streptococcal experiments have been tested in the manner just described. Either the results have been negative, or a doubtful positive reaction was noted in 1-10 dilution of serum. Additional evidence of

the improbability of agglutinins participating in the lethal action of the sera is furnished by the diverse types of infection which evoked the appearance in patients' blood of a common serological property.

In previous reports from this laboratory (4) an agglutination phenomenon obtained with hemolytic streptococci in patients' sera was reported which had several conditions differentiating it from true antibacterial agglutination, but which had certain factors in common with the streptococcal action of sera with which the present articles deal. The clumping of streptococci, just mentioned, occurred with serum from patients acutely ill but was not demonstrable after recovery. It was also noted that some strains of hemolytic streptococci formed aggregates under the conditions just described, but the majority of strains were not visibly affected. In order to determine whether the pseudo-agglutination reaction and killing power of patients' sera were causally related, comparable tests were carried out with strains of streptococci suitable for the two purposes. Since the results fail to reveal any clear cut relationship, it is unnecessary to present detailed protocols. It may be briefly stated that none of the three strains (Se, Co, and Ba) most commonly used in the streptococcal tests and differing in sensitiveness to the lethal action of sera, belong to the so called agglutinable group. Furthermore, two additional strains, which are susceptible to pseudo-agglutination under proper conditions, did not exhibit in streptococcal tests any special characteristics which separated them from other strains. Further investigation of this phase of the problem is now in progress.

DISCUSSION

In the first article of this series, results were presented which indicated that factors, *in vivo*, associated with acute illness are significant in determining the presence of a demonstrable streptococcal property in the serum of patients. In this, the second report, it is demonstrated that additional circumstances, *in vitro*, may be introduced which further affect the phenomenon. The conditioning elements may be contributed to some extent by the particular strain of hemolytic streptococcus employed in the test, but to a much more striking degree by the aerobic or anaerobic state of the environment in which the experiments were performed.

Information concerning differences in susceptibility among the strains is, at the present time, incomplete. A few strains have been found to be highly sensitive to the poisoning effect of patients' sera; a few others have been consistently the most resistant; the majority, however, have occupied a broad intermediate position between the two extremes. In view of the fact that the presence or absence of air has been found to be so significant in determining the death or survival of hemolytic streptococci in patients' sera, ones attention is directed toward the physiological processes of the organisms in which the availability or exclusion of oxygen may be concerned. Callow (5) found that, among twelve aerobic bacterial species, only streptococci were devoid of catalase; one of the strains used was *Streptococcus hemolyticus*. Farrell (6) confirmed this finding and, in addition, reported that neither cytochrome nor indophenol-oxidase could be demonstrated in streptococci. A thermostable peroxidase, having special characteristics, was, however, found to be present. McLeod and Gordon (7), in classifying bacteria according to sensitiveness to hydrogen peroxide, found the susceptibility of streptococci to be relatively high as compared with other aerobes, and there was also some difference between the strains of streptococci. Stevens (8) recently reported that irradiated cod liver oil and oil of pine are bactericidal for hemolytic streptococci. He ascribed the killing effect to peroxides which were formed in the oils as a result of ultra-violet radiation. He also reported that reduction of irradiated oil with cysteine hydrochloride inactivated the bactericidal process. When the observations reported in this article are considered in connection with the findings of others, which have just been outlined, it seems possible that the special biological reactions of *Streptococcus hemolyticus* pertaining to its respiratory mechanism and interrelated activities may be significant in the present study.

The fact that the streptococcal process in sera was inactivated by exclusion of air does not, however, answer the question of whether the effect of the anaerobic state is on the bacterial cells or on the bactericidal element in the serum. If the former interpretation is correct, then it must be presumed that biological processes of hemolytic streptococci proceed differently, depending on the presence or absence of air. Farrell (6) found that the uptake of atmospheric oxygen by

washed streptococci was negligible as measured by the Warburg apparatus. Unpublished observations¹ indicate that hemolytic streptococci live and multiply in atmospheres ranging from complete anaerobiosis to that of 95 per cent oxygen. Even though atmospheric oxygen does not appear to exert a direct effect on the viability of streptococci, indirect influences may be involved. For example, it is well known that some facultative anaerobic bacterial species utilize different metabolic processes under aerobic and anaerobic conditions and that the products of metabolism may also be different, depending on the availability or exclusion of air. It becomes necessary, therefore, to take the factors just mentioned into consideration in attempting to analyze the basic mechanism of the streptococcal reaction.

As suggested earlier, the other possible effect of anaerobiosis is upon the serum. If the differences in the outcome of experiments carried out under aerobic and anaerobic conditions are due to the effect of environment upon the streptococcal property of serum, then it may be surmised that the element in serum responsible for the death of streptococci is active in an oxidized form but seriously impaired when reduced. It is impossible at the present time to offer a correct interpretation of the results. However, in spite of the present limitations, the findings suggest that the killing power of patients' sera depends upon the presence of a property which interferes with some phase of streptococcal physiology necessary for the aerobic life of the bacterial cell. The results also emphasize the difficulty of attempting to correlate the streptococcal power of patients' sera *in vitro*, with activities *in vivo*, where factors pertaining to oxidative and reductive states in an infected individual are incompletely understood.

SUMMARY

Although sera derived from patients at the time of acute, active infection were found to be capable of destroying hemolytic streptococci under aerobic conditions, the organisms retained viability when the tests were performed in the environment of anaerobiosis afforded by a vaseline seal or an anaerobic jar.

Within the limitations of the experimental procedures which were

¹ Made in this laboratory by Dr. R. L. Garner and the author.

employed, the aerobic or anaerobic effect was found to be a reversible reaction.

Heating sera at 60°C. for 1 hour inactivated the streptococcidal element in most instances, but not in every case; heating at 56°C. for 1 hour impaired the killing power of half of the specimens which were tested.

Sera retained the capacity to destroy hemolytic streptococci when kept in the ice box for 3 weeks; a slight diminution in killing power was noted after 4 weeks.

By the methods which were employed, the streptococcidal property of sera could not be correlated with either true antibacterial agglutination or with pseudo-agglutination.

The significance of the findings as a basis for analyzing the mechanism of the streptococcidal phenomenon is discussed.

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THE PRODUCTION OF BLOOD PLATELETS IN THE LUNGS

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PLATE 6

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The present investigation had its origin in the suggestion that the blood of the emergent vein of the organ or organs in which platelet production is taking place should show a higher platelet count than the venous blood of other organs. In order to carry out this idea it was essential to find a satisfactory method of counting both platelets and erythrocytes in samples of blood removed from the veins by means of a syringe. Such a method was devised. It is described in detail below. In applying this method to various arteries and veins it was found that the platelet count, relative to the number of erythrocytes, was, in general, higher in the arteries than in the veins, indicating an increase in the number of platelets during the passage of the blood through the lungs. This point, therefore, was made the subject of a special study by experimental and histological methods, the results of which are given in this paper.

Methods

Removal of Samples of Blood.—For this purpose 0.1 cc. of blood was taken from an artery or vein by means of a 0.25 cc. tuberculin syringe (B-D). The syringes used were calibrated with mercury, but the graduation as given by the makers was found to be correct. In these syringes the 0.25 cc. is divided into 25 equal parts, each representing 0.01 cc. In taking a sample of blood the syringe with needle attached was filled with the diluting mixture to mark 15. The needle (No. 24) was then inserted into the vessel and blood was drawn to mark 25, giving, therefore, 0.1 cc. of blood. The contents of the syringe were emptied into 39 cc. of the diluting mixture and the syringe was filled and emptied three times with the diluting mixture, so that the final dilution of the blood was 0.1 cc. in 40 cc. of the mixture, or 1 to 400. This mixture was stirred thoroughly for 5 minutes with an electric stirrer, the wide paddles of which were coated with bakelite varnish, and samples were then taken for the counting chamber by means of an appropriate

capillary tube. A count was made first of the erythrocytes in 10 or 15 of the small squares (improved Neubauer ruling). This gave time for the platelets to settle and they were then counted in the entire central ruled square millimeter. In this way an absolute count was obtained of both the erythrocytes and the platelets, and the ratio of one to the other could be determined.

The Diluting Mixture.—Platelets are well preserved by a number of solutions, such as sodium metaphosphate or sodium citrate or fluoride, but for our purpose such solutions were not usable owing to the fact that in the counting chamber there was more or less rapid hemolysis of the erythrocytes, the "contact hemolysis" of Fenn. This hemolysis not only prevented accurate counting of the erythrocytes, which was desirable for our purpose, but interfered also with counting of the platelets, especially in the blood of the cat with its small erythrocytes, owing to the fact that particles given off from these latter or their "ghosts" simulated the appearance of platelets and made the count uncertain. The mixture which proved to be most satisfactory for our purpose in preserving both platelets and erythrocytes was a buffer solution of phosphates, pH 7.3, to which was added heparin dissolved in a 1.5 per cent solution of sodium chloride. We designate this mixture as a heparinized buffer solution. For the blood of cats and dogs the following formula was used.

NaCl, 1.5 per cent solution.....	50 cc.
Na ₂ HPO ₄ (11.876 gm. per liter).....	40 "
KH ₂ PO ₄ (9.078 " " ").....	10 "
Heparin (crude, Hynson, Westcott, and Dunning).....	60 mg.

The heparin is dissolved in the mixture of salts which is then filtered. The filtrate is boiled gently for a few minutes, cooled, and centrifugalized at high speed for 15 minutes in sterile centrifugal tubes. The solution decanted off is ready for use. Unless kept in the ice box it should be used within a short time since on standing bacteria develop. Suspended particles and bacteria interfere with the accuracy of the platelet count, hence the necessity of centrifugalizing the mixture and sterilizing the syringes and all the containers. The counting chamber should be cleaned carefully before using. We have found it best to wash thoroughly with water and then very freely with alcohol, using a cloth without lint. While ordinarily one can distinguish between platelets and foreign particles, or minute droplets adhering to the glass, yet contaminations of this kind or the presence of bacteria may make a count unsatisfactory or impossible. The diluting mixture may be kept in the ice box for long periods, but should be boiled and centrifugalized before using. It is quite possible to use the mixture two or more times. In this case the liquid with its contained sample of blood is centrifugalized, decanted, boiled, and again centrifugalized. Any loss of liquid is made good by the addition of water. If not used at once it should be kept in a refrigerator and just before using should again be boiled and centrifugalized. Stock mixtures of the saline and phosphates may be made and used over long periods. When counts were made upon human blood the formula was modified by using a 2 per

cent solution of sodium chloride in place of the 1.5 per cent solution, and substituting K_2HPO_4 for Na_2HPO_4 .

Histological Methods.—In studying platelet formation in the lungs and other tissues no success followed the use of material preserved in a Zenker mixture or in mercuric chloride or picroformol solutions. When the material was preserved in Dominici's solution and the sections were stained with a modified Wright's stain excellent results were obtained.¹ In fixing the lung tissue the animal was allowed to bleed to death and the Dominici solution was injected under low pressure either into the pulmonary artery or the trachea. Pieces of the tissue were then cut out and placed in the Dominici solution for $\frac{1}{2}$ hour, and then passed through graded alcohols, 50 to 100 per cent, imbedded in paraffin, and cut in sections of 5 to 7μ . The sections fixed on the slide were stained in the Wright's mixture according to the procedure below. To obtain a good differentiation of platelets and platelet material it was found desirable to treat the stained section for a brief time with very dilute acid. In a successful preparation the platelets stand out with great distinctness owing to the characteristic brilliant color of the contained granules. The steps in the process of staining the sections were as follows: (1) xylene; (2) butyl alcohol; (3) methyl alcohol; (4) Wright stain for 30 seconds; (5) add twice the amount of water and allow to stand under cover for 25 minutes; (6) wash in water; (7) immerse in dilute acid solution (3 drops glacial acetic acid to 500 cc. water) for 20 to 30 seconds, until blue coloring matter begins to come off in clouds; (8) immerse momentarily in alkaline solution (sodium bicarbonate 0.25 per cent) and then in water for 2 minutes. The specimen may now be passed through acetone and xylene and be mounted, but experience has shown that a better differentiation of platelets is obtained if the process is repeated. From the water the section is treated with methyl alcohol which decolorizes it more or less completely, and then steps 4, 5, 6, 7, and 8 are repeated. From the water the specimen, after removal of surplus water from the slide, is transferred to acetone, xylene, and mounted in balsam.

The Platelet Count in Arterial and Venous Blood

By the method described specimens of arterial and venous blood were taken as nearly simultaneously as possible from various arteries and veins, chiefly from the carotid and jugular, the femoral artery and vein, and the pulmonary artery and aorta at the base of the heart.

¹ The Dominici fixing solution was prepared according to the formula given in *Folia haematol.*, 1905, 2, 219. Downey's solution, 10 per cent formalin in physiological salt solution saturated with $HgCl_2$, also gives good results, but in our experience the platelet material does not stain so well as after Dominici solution. The Wright's stain used by us was prepared by Hynson, Westcott, and Dunning, Baltimore. This stain gave us better results than any other that was tried.

Cats were used in most of the experiments, anesthetized with ether, sodium luminal, or sodium amytal. The last named is preferable, using 60 to 100 mg. per kilo injected intraperitoneally. Counts were made independently by each of us. Successive counts on the same mixture of blood and diluting solution showed variations of greater or less magnitude. Some idea of the extent of these variations is given by the following series made upon a specimen from the femoral vein of a normal cat under ether. Before each count the mixture was stirred by the electric stirrer.

		Erythrocytes	Platelets
Observer 1	First count	9,730,000	288,000
	Second "	9,210,000	292,000
	Third "	9,560,000	292,000
	Average.....	9,500,000	290,000 Ratio 1:32.8
Observer 2	First count	9,910,000	296,000
	Second "	9,800,000	281,000
	Third "	9,800,000	282,000
	Fourth "	9,650,000	291,000
	Fifth "	9,480,000	288,000
	Average.....	9,740,000	289,000 Ratio 1:33.7

Averaging the two results the count would be recorded as erythrocytes 9,620,000, platelets, 289,500, giving a ratio of 1 platelet to 33.2 erythrocytes. When the technique is good the method gives concordant results, but in platelet counting the greatest source of error, in our experience, lies in the factors already referred to, namely, the presence of foreign particles, or air droplets on the surface of the counting chamber. When a specimen of this kind was encountered it was discarded and a new one was made. In comparing the arterial and venous counts dependence was placed upon the ratio of platelets to erythrocytes rather than upon the absolute counts, since in spite of the care taken to obtain equal specimens some slight variations in the amount of blood removed by the syringe were at times unavoidable.

Comparisons between arterial and venous blood in normal anesthetized cats were made in four series occurring at different times during the investigation, and amounting to a total of 89 animals. The figures for each of these cases are given for the first three series in order to illustrate the variations found in normal animals. When the ratio of platelets to erythrocytes in the companion artery and vein differed by less than one the count was considered as equal in the two vessels (see Table I).

TABLE I

Series I					Series I—Continued				
Experi- ment		R.B.C.	Platelets	Ratio	Experi- ment		R.B.C.	Plate- lets	Ratio
<i>1934</i>					<i>1934</i>				
Apr. 5	A. left aur. V. right aur.	7,640,000 7,150,000	554,000 479,000	1:13.8 1:14.9	June 20	A. aorta V. pulm. art.	7,708,000 7,618,000	579,000 472,000	1:13.3 1:16.1
" 9	A. sup. mes. V. portal	7,380,000 7,970,000	557,000 543,000	1:13.2 1:14.7	" 27	A. aorta V. pulm. art.	5,685,000 6,070,000	613,000 517,000	1:9.3 1:11.7
" 12	A. carotid V. portal	11,180,000 9,670,000	576,000 502,000	1:19.4 1:19.2	" 28	A. aorta V. pulm. art.	5,935,000 6,235,000	351,000 350,000	1:16.8 1:17.8
" 17	A. aorta V. splenic	8,170,000 8,050,000	510,000 460,000	1:16 1:17.5	Series II				
" 19	A. left aur. V. pulm. art.	7,310,000 7,270,000	475,000 427,000	1:15.4 1:17.0	<i>1934</i>				
May 3	A. left aur. V. pulm. art.	6,250,000 6,220,000	386,000 329,000	1:16.1 1:18.9	Oct. 5	A. carotid V. jugular	6,160,000 6,150,000	311,000 234,000	1:19.8 1:26.2
" 7	A. carotid V. jugular	6,720,000 6,750,000	756,000 530,000	1:8.8 1:12.7	" 5	A. carotid V. jugular	7,520,000 6,475,000	408,000 324,000	1:18.4 1:20.0
" 8	A. left. aur. V. pulm. art.	5,570,000 6,030,000	554,000 535,000	1:10.1 1:11.3	" 9	A. carotid V. jugular	11,290,000 10,250,000	454,000 470,000	1:24.8 1:21.8
" 9	A. carotid V. jugular	7,930,000 8,550,000	462,000 399,000	1:17.1 1:21.3	" 11	A. carotid V. jugular	7,170,000 6,850,000	366,000 351,000	1:19.3 1:19.5
" 14	A. carotid V. jugular	4,390,000 4,380,000	142,000 138,000	1:30.9 1:31.7	" 31	A. aorta V. pulm. art.	8,230,000 9,660,000	486,000 489,000	1:16.7 1:19.7
" 23	A. carotid V. jugular	5,950,000 5,730,000	295,000 258,000	1:20.1 1:22.2	Nov. 3	A. aorta V. pulm. art.	8,572,000 7,760,000	524,000 428,000	1:16.3 1:18.0
" 25	A. carotid V. jugular	8,947,000 8,405,000	735,000 595,000	1:12.1 1:14.1	" 6	A. carotid V. jugular	6,240,000 6,290,000	465,000 348,000	1:13.4 1:18.0
June 4	A. carotid V. jugular	11,220,000 10,260,000	333,000 316,000	1:33.6 1:32.1	" 8	A. aorta V. pulm. art.	8,010,000 7,990,000	528,000 416,000	1:15.2 1:19.2
" 7	A. aorta V. sup. mes.	8,950,000 8,940,000	337,500 343,000	1:26.5 1:26.1	" 12	A. aorta V. sup. mes.	9,060,000 9,330,000	626,000 526,000	1:14.4 1:17.7
" 11	A. aorta V. pulm. art.	9,000,000 8,897,000	262,000 238,500	1:34.3 1:37.3	" 14	A. aorta V. sup. mes.	7,630,000 6,580,000	572,000 500,000	1:13.3 1:13.1
" 12	A. aorta V. splenic	7,420,000 7,140,000	534,500 515,000	1:13.8 1:13.8	" 16	A. aorta V. pulm. art.	6,280,000 6,380,000	444,000 360,000	1:14.3 1:17.7
" 13	A. carotid V. jugular	7,626,000 7,726,000	1,001,000 902,000	1:7.6 1:8.6	" 20	A. aorta V. sup. mes.	9,360,000 8,930,000	506,000 398,000	1:18.5 1:22.4
					" 30	A. aorta V. pulm. art.	5,920,000 5,140,000	628,000 544,000	1:9.4 1:9.4

TABLE I—*Concluded*

Series III					Series III—Continued				
Experi- ment		R.B.C.	Platelets	Ratio	Experi- ment		R.B.C.	Plate- lets	Ratio
1935					1935				
Mar. 12	A. aorta	6,720,000	416,000	1:16.1	July 8	A. femoral	4,790,000	310,000	1:15.4
	V. pulm. art.	6,620,000	352,000	1:18.8		V. femoral	4,540,000	255,000	1:17.8
Apr. 1	A. aorta	4,060,000	138,000	1:29.2	" 15	A. femoral	10,450,000	271,000	1:38.5
	V. pulm. art.	4,200,000	142,000	1:29.5		V. femoral	10,150,000	253,000	1:40.1
June 29	A. femoral	9,860,000	370,000	1:26.6	" 22	A. femoral	5,840,000	317,000	1:18.4
	V. femoral	9,350,000	309,000	1:30.2		V. femoral	5,890,000	291,000	1:20.2
July 1	A. aorta	7,580,000	425,000	1:17.7	" 23	A. femoral	8,580,000	511,000	1:16.8
	V. pulm. art.	7,830,000	389,000	1:20.0		V. femoral	8,710,000	465,000	1:18.7
" 1	A. aorta	7,810,000	405,000	1:19.2	" 24	A. femoral	8,580,000	443,000	1:19.4
	V. pulm. art.	7,200,000	315,000	1:22.8		V. femoral	8,730,000	417,000	1:20.9
" 6	A. femoral	5,820,000	422,000	1:13.8					
	V. femoral	5,420,000	346,000	1:15.6					

The results show that of the total of forty-four observations thirty-four or 77.3 per cent gave a higher count of platelets in the artery than in the vein; eight or 18.2 per cent gave an equal count for artery and vein, and two or 4.5 per cent showed a higher count in the vein. It is probable that series III gives the most reliable count, since the conditions were more favorable and there had been greater experience with the technique. In this series only one of the eleven observations failed to show a larger number of platelets in the arterial blood.

Series IV was a miscellaneous group in which specimens were taken from artery and vein before or after various experimental procedures. Forty-five comparisons were made. Of these 75.5 per cent showed a larger platelet count in the artery. In 22.2 per cent the count was approximately the same in artery and vein. In one case only was the count larger in the vein.

We have not found in the literature any reference by other observers to a possible production of platelets in the lungs, except an interesting observation reported by Simpson. In her study of the experimental production of macrophages in the circulating blood she states that in certain cases, after the injection of colloidal solutions, there was an enormous reduction or practical disappearance of platelets in the blood of the right heart, while the blood of the left ventricle showed

the presence of a normal number. The author suggests that "a remarkable process goes on in the lung, whereby the distinctly abnormal venous blood is rendered normal by the time it reaches the left ventricle." We have tried to repeat this interesting observation upon cats by the use of India ink, given in single large doses or in smaller doses over a number of days, but so far have been unsuccessful. Hongo and Maéda in their paper upon hematopoiesis in the lungs report that in rabbits after intravenous injection of colloidal iodine or silver, especially if accompanied by hemorrhage, a condition of myeloid metaplasia develops in the lungs. Lung smears show the characteristics of marrow smears. They state that similar results were obtained by Iishimas. They make no reference, however, to platelet production.

Occasional references are found in the literature to comparative counts of platelets in arteries and veins, especially the splenic artery and vein in connection with the supposed function of the spleen as a destroyer of platelets. The general view has been that the splenic vein contains fewer platelets (Galloway). The only report of comparative observations on a larger scale that we have been able to find is in the paper published by Holloway and Blackford. They report experiments upon some twenty dogs in which comparisons were made between the splenic artery and vein, the jugular and carotid, and the femoral artery and vein. With few exceptions their counts showed a larger number of platelets in the veins, a result just the reverse of ours. But it may be said that the method used by them to get their specimens of blood could scarcely be expected to give accurate results for platelets. They punctured the artery or vein and took specimens from the blood as it flowed out. Some of their results showed differences that in themselves would seem to indicate the inadequacy of the method, e.g., femoral artery, 76,000, femoral vein, 342,000.

Comparison of Counts in Artery and Vein under Conditions Causing a Reduction in Platelets.—The conclusion suggested by the above observations on normal animals is strengthened by the results of experiments in which the number of circulating platelets was first reduced by various methods. The most direct of these methods, perhaps, is the one used by Duke in which the animal is bled repeatedly in small lots and the blood after defibrination is reinjected. Cats upon which this operation had been carried out were examined in some cases shortly after the operation, in other cases 1 day, 3, and 5 days after the operation. Brief reports are given of the results obtained (see Table II).

In all of these experiments the number of platelets in the artery was distinctly greater than in the vein. The difference was most marked so far as the ratio is concerned when the blood was examined

shortly after the operation or within 24 hours, owing to the reduced number of platelets. After 3 to 5 days when the regeneration of the platelets, which Bunting has shown precedes that of the erythrocytes, had made considerable progress toward normal, the difference between artery and vein approached that found under normal conditions.

TABLE II
Defibrination

	Time after bleeding		R.B.C.	Platelets	Ratio
(1) Oct. 9, 1934. Cat, bled from carotid, 4 lots of 25 cc. each. Blood defibrinated and reinjected	Before bleeding, jugular		10,250,000	470,000	1:21.8
	After bleeding $1\frac{1}{2}$ hr.	Artery	9,590,000	294,000	1:32.6
		Vein	9,470,000	228,000	1:41.4
(2) Oct. 23, 1934. Cat, bled from carotid, 4 lots of 25 cc. each. Blood defibrinated and reinjected	Before bleeding, jugular		6,385,000	393,000	1:16.2
	After bleeding $1\frac{1}{2}$ hr.	Artery	9,430,000	298,000	1:31.6
		Vein	9,230,000	244,000	1:37.8
(3) Oct. 25, 1934. Cat, bled from carotid, 7 lots of 25 cc. each. Blood defibrinated and reinjected	Before bleeding, jugular		7,600,000	536,000	1:14
	After bleeding $1\frac{1}{2}$ hr.	Artery	7,235,000	228,000	1:31.7
		Vein	7,900,000	202,000	1:39.1
(4) Feb. 5, 1935. Cat, bled from carotid, 7 lots of 25 cc. each. Blood defibrinated and reinjected	After bleeding 24 hrs.	Artery	5,600,000	112,000	1:50
		Vein	5,650,000	88,000	1:64.5
(5) Feb. 8, 1935. Cat, bled from carotid, 4 lots of 50 cc. each. Blood defibrinated and reinjected	Before bleeding, jugular		9,026,000	480,000	1:18.7
	After bleeding 72 hrs.	Artery	6,360,000	282,000	1:22.5
		Vein	6,200,000	256,000	1:24.2
(6) Feb. 15, 1935. Cat, bled from carotid, 4 lots of 50 cc. each. Blood defibrinated and reinjected	Before bleeding, jugular		9,820,000	210,000	1:47
	End of bleeding, carotid		7,840,000	56,000	1:140
	After bleeding 120 hrs.	Artery	5,860,000	292,000	1:20
(7) Mar. 27, 1935. Cat, bled from carotid, 4 lots of 50 cc. each. Blood defibrinated and reinjected		Vein	5,580,000	240,000	1:23.2
	Before bleeding, jugular		9,390,000	680,000	1:13.8
	End of bleeding, carotid		6,440,000	228,000	1:28.3
(8) May 8, 1935. Cat, bled from femoral, 5 lots of 50 cc. each. Blood defibrinated and reinjected	After bleeding 120 hrs.	Artery	5,710,000	442,000	1:12.9
		Vein	5,360,000	348,000	1:15.4
	Before bleeding, femoral v.		8,500,000	362,000	1:23.4
	End of bleeding, femoral a.		7,040,000	124,000	1:56.8
	After bleeding, 24 hrs.	Artery	5,580,000	202,000	1:26.9
		Vein	5,800,000	150,000	1:38.6

In another series the platelets were reduced by the intravascular injection of saponin (Table III). The actual effect of the saponin on the platelets was not determined since in these experiments a preliminary blood count was omitted. It would seem, however, from the figures, that it caused some reduction in platelets, except in the experi-

ment of June 25, in which, in spite of the fact that a large dose was given, the number of platelets appears to have been increased. In all

TABLE III
Saponin Injections

	Time after injection		R.B.C.	Platelets	Ratio
(1) May 1, 1934. Cat, saponin, intravenous, 0.75 mg. per kilo	24 hrs	Artery Vein	6,930,000 7,140,000	182,000 123,000	1:38.3 1:58
(2) May 10, 1934. Cat, saponin, intravenous, 1 mg. per kilo	24 hrs.	Artery Vein	7,962,000 8,185,000	257,000 209,000	1:31 1:39.1
(3) May 14, 1934 Cat, saponin, intravenous, 0.75 mg. per kilo	40 min.	Artery Vein	4,750,000 4,380,000	152,000 127,000	1:31.2 1:43.4
(4) June 4, 1934. Cat, saponin, intravenous, 0.33 mg. per kilo	24 hrs	Artery Vein	9,000,000 9,663,000	360,000 266,000	1:25 1:36.3
(5) June 25, 1934. Cat, saponin, intravenous, 1.25 mg. per kilo	24 hrs.	Artery Vein	5,283,000 5,755,000	649,000 572,000	1:8.1 1:10.1

TABLE IV
Peptone Injections

	Time after injection		R.B.C.	Platelets	Ratio
	min.				
(1) June 21, 1934. Cat, peptone, intravenous, 0.3 gm. per kilo	10	Artery Vein	8,070,000 8,490,000	82,000 69,000	1:98.4 1:123
(2) June 22, 1934. Cat, peptone, intravenous, 0.3 gm. per kilo	10	Artery Vein	8,920,000 9,250,000	249,000 189,000	1:35.8 1:48.9
(3) July 2, 1934. Cat, peptone, intravenous, 0.3 gm. per kilo	25	Artery Vein	10,825,000 10,540,000	270,000 234,000	1:40.1 1:45
(4) Oct. 10, 1935. Cat, peptone, intravenous, 0.3 gm. per kilo	10	Artery Vein	8,200,000 8,620,000	76,000 54,000	1:108 1:160
(5) Nov. 20, 1935. Cat, peptone, intravenous, 0.3 gm. per kilo	5	Artery Vein	4,500,000 4,240,000	110,000 80,000	1:41 1:53
(6) Feb. 19, 1936 Cat, peptone, intravenous, 0.3 gm. per kilo	5	Artery Vein	9,410,000 9,610,000	166,000 140,000	1:56.7 1:68.6

of the experiments the arteries were found to contain more platelets than the veins and the difference, as regards the ratio, was greater,

except in the experiment of June 25, than was found under normal conditions.

In a third series the platelets were reduced by means of a peptone solution made up in 0.9 per cent sodium chloride and injected intravenously in amounts equal to 0.3 gm. of peptone per kilo of animal. In these cases the blood was examined shortly after the injection, since, as Pratt and others have shown, the effect of peptone upon the number of platelets is only transitory, largely disappearing within an hour or two (Table IV).

Comparison of Arterial and Venous Blood of Special Regions.—The prevalent view that platelets are formed in the bone marrow made it desirable to examine the blood of the emergent veins of the long bones, but on account of their small size in the cat this was not practicable for the method we were using.

Vena Azygos: The vena azygos, however, which brings back blood from the ribs and other tissues is of large size, and it would seem probable that if the red marrow is the sole source of platelet production the blood of this vein should show a larger number of platelets compared with other veins, or the arteries. In the observations made upon this vein the thorax was opened along the mid-line of the sternum, the two lowermost ribs on the right side were cut away, and the vein was ligated just above the diaphragm. A specimen was then taken from the vein where it bends over to join the superior cava. Ten such experiments were made. In nine of the ten experiments the ratio of platelets to erythrocytes was lower in the blood of the vena azygos than in that of the left auricle or aorta. In one the ratio was the same. *Hepatic Vein:* In the experiments made upon this vein the inferior cava was ligated below the diaphragm. The thorax was then opened and a specimen of blood was taken from the cava above the diaphragm. The blood count from it was compared with that of a specimen from the portal vein taken either before or after the ligation of the inferior cava below the diaphragm. Seven observations were made. In five of them the platelet count was lower in the hepatic blood than in the portal blood. In the other two cases the platelets in the hepatic blood were slightly in excess, as judged by their ratio to the erythrocytes. The evidence, therefore, indicates that the blood loses rather than gains in platelets in passing through the liver. *Splenic Vein:* Specimens of splenic blood were taken either from the main trunk where it joins the mesenteric vein or from one of the smaller veins near its emergence from the spleen. They were compared with specimens of arterial blood taken from the abdominal aorta. Eleven observations were made. In ten of them the platelet count was lower in the splenic vein than in the aorta. In the remaining case the count was approximately identical. Here also the evidence indicates a loss of platelets in the blood passing through the spleen. *Superior Mesenteric Vein:* Comparative counts were made in ten cases

of specimens of blood taken from the superior mesenteric vein and the abdominal aorta. In seven of them the platelet count in the vein was lower and in three the ratios were practically the same, indicating the probability that the blood loses platelets in passing through the capillaries of the intestine.

Observations upon Human Blood.—It is desirable, of course, to extend these comparative counts to animals other than the cat, and, especially, to ascertain whether a similar difference holds for human blood. Some few observations of this kind that we were able to make may be referred to briefly in this connection. It happens that in one of the authors the radial artery of one wrist is easily accessible and several counts were made upon specimens taken from this artery compared with venous blood from the median cubital vein at the elbow. Two or more counts were made by each observer and the averages were used in making the comparisons.

		Erythrocytes	Platelets	Ratio
<i>1934</i>				
Feb. 19	Artery	5,480,000	274,000	1:20
	Vein	5,650,000	232,000	1:24.3
" 23	Artery	5,016,000	255,000	1:19.7
	Vein	5,451,000	245,000	1:22.2
Mar. 16	Artery	5,347,000	306,000	1:17.4
	Vein	5,610,000	278,000	1:20.2
Apr. 11	Artery	5,202,000	257,000	1:20.2
	Vein	4,843,000	240,000	1:20.1
" 27	Artery	5,751,000	255,000	1:22.5
	Vein	5,702,000	255,000	1:20.0
June 8	Artery	5,750,000	295,000	1:19.1
	Vein	5,270,000	192,000	1:27.4
<i>1935</i>				
July 3	Artery	5,360,000	264,000	1:20.3
	Vein	6,055,000	250,000	1:24.2
" 22	Artery	5,840,000	317,000	1:18.4
	Vein	5,890,000	291,000	1:20.2
<i>1936</i>				
* Mar. 13, 11 a m	Artery	5,850,000	192,000	1:30.4
	Vein	5,380,000	167,000	1:32.2
" 13, 3 p m.	Artery	5,400,000	156,000	1:34.6
	Vein	5,216,000	137,000	1:38.0

* The last two counts were made in connection with an experiment on the effect of occlusion of the veins. The subject was not well at the time and was subsequently sent to the hospital. This fact probably accounts for the unusually low platelet count.

In eight of the ten examinations the number of platelets was larger in the artery, in one the count was practically the same, and in one it was slightly larger for the vein. If one takes the average of the ten observations, as seems to be permissible, since they were made upon

TABLE V
Thrombocytopenia

		R.B.C.	Plate-lets	Ratio
(1) Jan. 24, 1931. Female, white, 5 yrs., diagnosis, purpura hemorrhagica. At time of observation patient much improved	Artery	4,050,000	278,000	1:15
	Vein	4,000,000	118,000	1:34
(2) Oct. 23, 1935. Male, white, 44 yrs., diagnosis, chronic purpura	Artery	4,630,000	139,500	1:33.2
	Vein	4,640,000	87,000	1:53.3
(3) Nov. 18, 1935. Male, white, 7 yrs., diagnosis, thrombocytopenic purpura	Artery	4,480,000	74,000	1:60.5
	Vein	4,860,000	50,000	1:97.2
(4) Feb. 4, 1936. Female, white, 2 yrs., diagnosis, thrombocytopenic purpura. Shortly after this observation the patient had a streptococcus infection, throat. Following this there was a long lasting rise in the platelet count as follows:	Artery	4,300,000	46,000	1:93.4
	Vein	4,600,000	20,000	1:230
Feb. 22, 1936	Artery	4,360,000	492,000	1:8.8
	Vein	4,200,000	400,000	1:10.5
Mar. 11, 1936	Artery	4,150,000	426,000	1:9.7
	Vein	4,380,000	368,000	1:11.9
June 10, 1936	Artery	4,280,000	153,000	1:27.9
	Vein	4,600,000	141,000	1:32.6
(5) Dec. 7, 1935. Male, white, 33 yrs., diagnosis, hemophilia? coagulation time 26 min.	Artery	6,080,000	96,000	1:63
	Vein	6,070,000	64,000	1:95.4
(6) Oct. 26, 1935. Male, negro, 50 yrs., diagnosis, aplastic anemia	Artery	1,110,000	8,600	1:128
	Vein	1,190,000	6,800	1:175
(7) Feb. 8, 1936. Male, negro, symptoms, nasal bleeding, swollen knee	Artery	4,300,000	307,000	1:14.4
	Vein	4,930,000	260,000	1:18.9
2nd examination Feb. 12, 1936	Artery	4,350,000	231,000	1:18.8
	Vein	4,410,000	197,000	1:22.3
(8) Feb. 18, 1936. Male, white, 6 yrs. Previous thrombocytopenic patient. Discharged Nov., 1933. Admitted for reexamination	Artery	5,040,000	372,000	1:13.5
	Vein	5,350,000	390,000	1:13.7

the same individual under like conditions, we get the following figures:

Artery, erythrocytes 5,499,600, platelets 257,100, ratio 1:21.4
 Vein, " 5,446,700, " 228,700, " 1:23.8

which indicate a difference quite comparable to the average result of the observations made upon the cat.

Several observations were made upon patients in the Johns Hopkins Hospital showing hemorrhagic conditions, chiefly cases with thrombocytopenia or purpuric symptoms. When there was distinct thrombocytopenia the difference in count between artery and vein was quite marked, but the figures, as far as they go, do not support the view of those who have suggested that the low count is due to increased destruction of platelets rather than to diminished production. The specimens of blood were taken for us by different members of the hospital staff, arterial blood from the radial artery and venous blood from the median cubital vein (Table V). With the exception of the last case the platelet count in the artery was larger than that in the vein.

Perfusion Experiments

In their experiments upon perfusion of the marrow of the tibia with oxalated saline, Drinker and Drinker comment upon the fact that in the perfusate no platelets were found. Following this suggestion a series of experiments was carried out in which the lungs and other organs were perfused with a platelet-preserving solution, and the perfusate was examined at certain intervals for its content in platelets and erythrocytes, and the proportion of one to the other.

The lungs were perfused through the heart. An inflow cannula was placed in the superior cava and an outflow cannula in the aorta at its origin from the heart, and the vena azygos and inferior cava were ligated. The perfusing fluid used in all the experiments was the saline phosphate, pH 7.3, described above in the method for making platelet counts, but without the heparin. In some experiments the perfusion was continuous, in others a certain volume was recirculated for a time and was then followed by a continuous perfusion. It was found that this solution sooner or later set up an edema of the lungs which terminated the experiment. In the later experiments this difficulty was avoided by the introduction of a certain amount of egg albumin in the perfusing liquid. The usual procedure in these cases was first to wash out most of the blood by passing through 200 to 300 cc. of the saline phosphate mixture and then to substitute the albuminous mixture, a definite volume of which was recirculated ten to twelve times. This was followed by a continuous perfusion of the saline phosphate mixture until edema began to develop. Samples of the perfusate were removed at regular intervals, heparin was added, and the sample was put aside for examination at the end of the perfusion. At the examination each sample was mixed thoroughly by an electrical stirrer and from the mixture a specimen was taken by means of a capillary tube to fill the counting chamber. Counts were made usually for the entire ruled area of the chamber.

In the first series of experiments, in which the saline phosphate mixture was used without the addition of albumin, comparative tests were made upon the lungs, liver, kidney, intestines, marrow, spleen, and pancreas. The results indicated clearly that in the lungs continuous perfusion brings out relatively more platelets, that is to say, the ratio of platelets to erythrocytes increased, as the perfusion continued, much more markedly in the lungs than in the other organs. A brief record of one experiment will indicate the character of the results obtained.

Nov. 16, 1934. Cat, 3 kilos, under luminal. After opening the chest specimens of arterial and venous blood were examined to determine the normal ratio of platelets and erythrocytes.

Artery, aorta, erythrocytes 6,280,000, platelets 444,000, ratio 1:14.3
 Vein, pulmonary artery, " 6,380,000, " 360,000, " 1:17.7

Perfusion was established through the lungs and the effluent was collected in five lots, each divided into four parts of 50 to 100 cc., as follows, A-1 to A-4, 400 cc.; B-1 to B-4, 200 cc.; C-1 to C-4, 200 cc.; D-1 to D-4, 200 cc.; E-1 to E-4, 400 cc. Total perfusate was 1400 cc. An interval of 5 minutes was given between the collection of each lot. Artificial respiration was maintained throughout the experiment.

Counts	Erythrocytes	Platelets	Ratio
A-1	470	65	1:6.3
A-4	142	37	1:3.8
B-1	231	55	1:4.2
B-4	411	113	1:3.6
C-1	793	373	1:2.1
C-4	161	113	1:1.4
D-1	106	80	1:1.3
D-4	71	61	1:1.1
E-1	172	170	1:1
E-2	65	80	1.2:1
E-4	27	49	1.8:1

The figures show that as the washing out proceeded the number of erythrocytes and platelets in the effluent varied irregularly, the erythrocytes showing a decreasing trend. The ratio of platelets to erythrocytes in the circulating blood before perfusion was approximately 1 to 16, but this ratio increased more or less steadily in the effluents until at the end there were nearly two platelets to each erythrocyte. The result would seem to indicate some kind of a supply of platelets in the lungs.

Similar experiments made upon other organs gave a different result. For example, *kidney*, total perfusion 1.5 liters. Initial ratio of platelets to erythrocytes

in the circulating blood 1 to 13. During the perfusion this ratio varied irregularly between 1 to 8 and 1 to 35, but at the end of the perfusion was 1 to 10.

Spleen and Pancreas (and Part of Stomach).—Total perfusion 2 liters. Initial ratio of platelets to erythrocytes in the circulating blood 1 to 20. During the perfusion the ratio varied irregularly between 1 to 13 and 1 to 29. In the last perfusate it was 1 to 19.5.

Intestines.—Total perfusion 2 liters. Initial ratio of platelets to erythrocytes in circulating blood 1 to 30. During the perfusion the ratio varied between 1 to 15 and 1 to 7. In the last perfusate it was 1 to 18, but in the last three samples collected the platelets were so few in number, one or none in the entire ruled field of the counting chamber, that the ratio lost its significance. The important fact, however, was the small number of platelets washed out of the tissue compared with the lungs.

Tibia.—The intention in this case was to perfuse the marrow of the tibia. The inflow cannula was inserted into the popliteal artery above the knee joint, and an attempt was made to ligate all branches except those entering the tibia. This was not successful as was evident from the volume of the outflow, so that the experiment gave the result of a perfusion of the marrow together with some of the muscles and tissue surrounding the tibia. Total perfusion something less than a liter. Initial ratio of platelets to erythrocytes in the circulating blood 1 to 20. During perfusion this ratio varied between 1 to 16 and 1 to 7.5. In the final effluent the ratio was 1 to 15, but here again the platelets toward the end of the perfusion were so few in number that a small difference in the count made a large change in the ratio.

Liver.—Total perfusion 1½ liters. Inflow cannula in portal vein. Initial ratio of platelets to erythrocytes in circulating blood 1 to 17. During perfusion the ratio varied between 1 to 15.5 and 1 to 6. In the final perfusate the ratio was 1 to 8.

In the second series of experiments the perfusion fluid containing albumin was employed. As stated above the organ was first washed out with the saline phosphate mixture. This was then replaced by the albumin mixture, 100 to 150 cc. of which was recirculated ten or more times. This was followed by a continuous perfusion of several hundred cc. of the saline phosphate mixture. Artificial respiration was maintained throughout the experiment. The results in the case of the lungs were striking as may be seen from the data of two experiments. Dec. 3, 1934. Initial perfusion with saline phosphate mixture 500 cc. Perfusate collected in five lots, B-1 to B-5, of 100 cc. each.

	Erythrocytes	Platelets	Ratio
B-1	511	56	1:9.1
B-3	535	154	1:3.4
B-4	367	207	1:1.8
B-5	227	133	1:1.7

The albumin-containing solution, 150 cc., was recirculated ten times and was followed by a continuous perfusion of the saline phosphate mixture, 150 cc., the effluent being collected in two lots, X and Y, each 75 cc.

	Erythrocytes	Platelets	Ratio
X	146	319	2.2:1
Y	46	177	3.8:1

Jan. 2, 1935. Lung. Initial perfusion with saline phosphate mixture 200 cc. Followed by recirculation of the albumin mixture, 150 cc., ten times. Then continuous perfusion of the saline phosphate, 850 cc. This perfusate was collected in seventeen lots of 50 cc. each. B-1 to B-17. Total perfusion 1200 cc.

	Erythrocytes	Platelets	Ratio
B-1	310	127	1:2.6
B-3	29	75	2.6:1
B-5	16	78	4.8:1
B-7	33	81	2.15:1
B-9	14	39	2.7:1
B-11	4	35	8.7:1
B-13	6	30	5:1
B-15	8	38	4.6:1

The results of these two experiments seem to indicate that a source of platelets exists in the lungs other than can be explained by the content of the circulating blood. A similar experiment was carried out with the liver. While the results showed a decided increase in the platelet ratio the increase was not comparable to that exhibited by the lungs.

Jan. 8, 1935. Liver. Perfusion through portal vein. Inferior cava ligated below diaphragm. Aorta and branches arising from it below diaphragm ligated. Initial perfusion with saline phosphate mixture 300 cc. Followed by recirculation ten times of the albumin solution and a final continuous perfusion of 1600 cc. of the saline phosphate solution. Effluent of the last perfusion collected in 100 cc. lots, C-1 to C-17. Total perfusion 2150 cc.

	Erythrocytes	Platelets	Ratio
C-1	269	61	1:4.7
C-3	268	71	1:3.7
C-5	233	48	1:4.8
C-7	131	30	1:4.4
C-9	50	11	1:4.5
C-11	24	8	1:3
C-13	34	11	1:3.1
C-15	25	7	1:3.6
C-17	73	22	1:3.3

Histology of Platelet Formation in the Lungs

Our experimental results indicated that platelet formation takes place in the lungs, and a histological study of the lungs was made by means of sections and smears to determine if possible the source and mode of production of the platelets.

Lung Smears.—Smears or impressions were made from a freshly cut surface of the lungs in normal animals and in animals in which the process of platelet formation had been accelerated by previous experimental reduction of the number of platelets in the circulating blood. The smears after drying were stained after the manner of blood smears in Wright's stain. These preparations while interesting were not very informative in regard to the origin of the platelets. In many of the slides numerous platelets were found, singly or in small or large groups, clearly marked out by their characteristic staining. They varied greatly in size and form, from the typical small platelet with a diameter of 2 to 4 μ to large forms the size of an erythrocyte or much larger. In many cases platelet masses occurred, elongated or oval or circular in form. These masses showed the characteristic staining of platelets, namely, a bluish ground substance thickly dotted with red-purple granules.

The lung smears showed numerous so called dust cells and not infrequently some of these were found to contain one or more platelets, but after careful study of many preparations we were convinced that this represented merely an occasional ingestion of platelets by these phagocytic cells and had no bearing upon the problem of platelet production.

Lung Sections.—The lungs were preserved in Dominici's solution, injected through the pulmonary artery or trachea, and were stained with Wright's stain, according to the method previously described. In such specimens platelets are found in numbers lying in the capillaries, singly or in groups, and also in the larger vessels scattered among the red corpuscles. They are very distinct on account of their characteristic staining. One cannot overlook them or mistake them for any of the other elements present. In addition one finds here and there in the preparation, in amounts that vary with conditions, what may be called platelet masses or platelet processes containing the characteristic red-purple platelet granules. These processes are of all sizes and shapes. Sometimes small oval bodies that might be designated as giant platelets, but frequently very large masses, or long strings lying in the capillaries (Fig. 7).

These processes or masses are not agglutinated platelets. The platelet granules are dispersed uniformly and not gathered into groups, as in the case of masses of agglutinated platelets. Their histology and the results of our examinations indicate that they are cast off pseudopodia or processes from the cytoplasm of the giant cells. They form a striking feature in sections of the lungs, particularly in the lungs of animals in which the process of platelet production has been accelerated by experimental methods, but they are present also in the lungs of normal animals, sometimes scanty, sometimes quite abundant. Their presence seems not to have been noted by most writers, but Bunting (1911) calls attention to their occurrence in the lung of the rabbit, under conditions of accelerated platelet production, as also in the circulating blood in Hodgkin's disease. In the marrow or, under some conditions, in the spleen similar detached platelet processes may be seen, but usually in the immediate neighborhood of a megacaryocyte, so that there may be a question as to whether they are really thrown off pseudopodia from a giant cell or whether the plane of the section has been such as to cut them off from the cytoplasm of the cell. In the lungs this doubt does not arise, the processes lie free in the blood vessels wholly separate from the giant cells.

The Giant Cells, Megacaryocytes, of the Lungs

The presence of giant cells in the lungs has been noted by many authors since they were first described and discussed by Aschoff. In most cases they are described as naked and degenerating nuclei without cytoplasmic material, although Aschoff himself speaks of the occurrence of some cells with surrounding cytoplasm, and Bianchini also refers to the presence of megacaryocytes with surrounding cytoplasm filled with azurophilic granules. The general opinion, however, has been that the giant cells in the lungs are migrants from the bone marrow (or spleen) which have lost their cytoplasm in the process of platelet formation before reaching the lungs, or in any case shortly after being caught in the lung capillaries, so that they appear usually as naked and degenerating nuclei, exhibiting evidence of pycnosis. The results of our study lead us to an entirely different view.

In the lung of the normal animal (the cat was used chiefly in our observations although corroborative results were obtained from the dog) giant cells are found in the sections in all cases, but in variable numbers, depending on the animal or possibly the luck of the section. In some cases in the normal cat they were quite numerous, as many, for example, as forty to an area of 100 sq. mm. Under experimental conditions, which caused an accelerated production of platelets, the number of giant cells in the lungs might be at least four times as great. They differ greatly in appearance. Some of them, perhaps most of them under ordinary

conditions, are the so called naked nuclei with no appearance of cytoplasm. Petri contends that they always show a small protoplasmic non-granular zone, sharply bordered, and in our preparations there seemed to be at times a narrow basic rim of cytoplasm, and in cases when a nucleus could be followed through serial sections, what seemed to be a naked nucleus might be found to have cytoplasmic process attached to some part of its periphery. In very many cases what would ordinarily be called a naked nucleus showed caps of cytoplasmic material at the ends of the elongated or branched nucleus, the caps being composed of platelet material containing purplish red granules which stood out in marked contrast to the blue nucleus. It seems probable that with stains that do not bring out the platelet granules these short processes of cytoplasm would be overlooked. In some cases nuclei of this type predominated and gave the impression of cells that might be in a state of active production of platelet material, the processes being cast off rapidly to form platelets.

In addition to this type of giant cell one finds in the lung of the normal animal and especially in the lung of animals in which platelet production had been accelerated, giant cell nuclei with one or more cytoplasmic processes attached, the processes being composed entirely of platelet material with its characteristic granules (Figs. 1 to 6). The processes varied greatly in size and shape and number; sometimes a single finger-shaped projection from one pole of the nucleus, or extending out from each of the several poles of a branched nucleus, sometimes very massive processes that entirely filled the capillary or small vessel in which they lay, and at other times a long branching process that penetrated the capillaries for a considerable distance, showing constrictions and swellings along its course. Owing to the frequently twisted shape of the nucleus and the projection of the cytoplasmic processes in different planes, it would be more or less a happy accident to get a section through any one giant cell of this character that would show its entire outline. Some of these cells show a quite extraordinary wealth of cytoplasmic processes pushing through the capillaries in many directions. As stated previously these processes are made up entirely of platelet material and are identical in appearance with the platelets themselves, and with the detached processes or masses found scattered through the blood vessels of the lungs. There would seem to be no doubt that these latter are broken off or detached processes of the giant cells and that by constriction or fragmentation they give rise to platelets. In general the giant cells of the lungs are different in appearance from the typical megacaryocyte with its crown or basket nucleus as found in the marrow or spleen. It is very rare, indeed, to find one with the nucleus placed centrally in the cytoplasm and showing a distinct lobulated or polymorphic appearance. Usually the nucleus is an elongated oval or is pulled out into a Y or branched form, and the cytoplasm springs from the ends or from separate points along the sides. The nucleus sometimes shows evidence of a polymorphic form but in other cases appears like a greatly enlarged single nucleus. In our preparations these nuclei do not show any indication of degeneration. While staining somewhat deeply

throughout with the basic dye (blue) there is no indication of pycnosis. We have seen distinctly degenerating nuclei with pycnotic staining more frequently in the giant cells of the marrow than in those of the lungs.

The giant cells of the lungs whether naked or with cytoplasmic processes have the appearance of active growing cells, and there can be no doubt that the cytoplasm consists of platelet material and gives origin to circulating platelets. The figures that we have reported in regard to the number of platelets in arterial and venous blood would seem to show that platelets are produced in the lungs. Granting that the blood does receive an accession of platelets in passing through the lungs, the histological examination gives no indication of a source of this platelet production other than from the cytoplasm of the giant cells.

The Origin of the Megacaryocytes of the Lungs

The prevalent view is that first suggested by Aschoff, namely, that the giant cells of the lungs are migrants from the bone marrow, or in the newly born or very young animal from the spleen. They are supposed to escape into the circulation and become trapped in the capillaries of the lungs. No very good direct proof has been furnished for this view. The possibility, however, that such a migration may occur is indicated by the fact that in a variety of pathological conditions, especially in myelogenous leukemia, megacaryocytes are found in the circulating blood. It is stated also that conditions, experimental or pathological, which tend to increase the number or activity of the megacaryocytes of the marrow bring about a parallel increase in the giant cells of the lungs. This, however, is not conclusive proof of the migration theory, since it seems obvious that conditions which stimulate the multiplication or functional activity of megacaryocytes might affect those in the lungs as well as those in the marrow. In sections of the marrow or the spleen one may find occasionally a mature megacaryocyte lying free in a large vein, in a position to be swept on into the circulation, and this fact may be accepted as indicating the possibility of an escape of these cells from the marrow or spleen, although in itself it can scarcely serve as a demonstration that all the megacaryocytes of the lungs have such an origin. It may be said, however,

that if this origin is accepted it does not follow that the giant cells of the lungs are on the way to degeneration and play no functional rôle. Our observations indicate on the contrary that in their new environment they are active in the production of platelets and give no indication of degenerative changes. The histological evidence for their participation in platelet formation is in fact more convincing than in the case of the marrow. So called Wright's figures are not always conspicuously in evidence in sections of the marrow, whereas in the lungs giant cells found with cytoplasmic processes are quite obviously engaged in the manufacture of platelets.

The alternative view to the migration theory is that the megacaryocytes are formed or developed in the lungs themselves. Some authors have taken this position. The results of our studies lead us to adopt this view, since the number of giant cells found at times in the lungs, and their evident activity in the production of platelets, hardly seems consistent with the theory that they are degenerating cells that have been cast off from the marrow.

It is usually assumed that the lung capillaries have a special tendency to hold back foreign material in the circulation, and would, therefore, be likely to filter out giant cells, if present. This belief was not supported by direct experiments. Freshly prepared emulsions of bone marrow were injected into the pulmonary artery, or one of its branches. On subsequent examination of the lung tissue, treated in this way and preserved and stained by the methods described, we could not satisfy ourselves that there was any increase in the number of megacaryocytes present, and those that were found did not exhibit the characteristics of the marrow cells with their distinctive nucleus and surrounding cytoplasm; they all belonged to the type usually present in the lungs.

The origin of the megacaryocyte is a much discussed question concerning which many different opinions have been expressed. Our study of the cell as it is found in the lungs, marrow, spleen, and liver at different ages and under different conditions has led us to accept the view proposed by Naegeli that they develop from a myeloblastic cell, which in its youngest stage possesses a clear nucleus with a conspicuous nucleolus and a narrow rim of cytoplasm that takes a deep basic stain with the Wright mixture. One finds in sections what seem

to be transitional forms between this cell and the young or immature form of the megacaryocyte with its large nucleus, single or lobulated, and its small zone of non-granular basic cytoplasm. In sections of the lungs, especially in those animals in which platelet production has been stimulated by defibrination of the blood or by the injection of peptone solutions, similar myeloblastic or lymphoblastic cells are found in numbers. They lie free in the capillaries much as they do in the liver capillaries of the fetus. They exhibit a great variety in size and shape. Transitional forms or what seem to be transitional forms between it and the giant cell of the lung may be found in which the nucleus is greatly enlarged and the cytoplasm begins to show the appearance of granules and takes a purplish stain. The origin of these myeloblastic cells is not evident. They are found in connection with megacaryocytes in the liver of the fetus, in the spleen, the marrow, and the lungs. When conditions are such as to lead to an increase in the number of megacaryocytes in the lungs there is a corresponding increase in number of these cells. In the lung of the embryo cat near term, or in the newly born kitten, when giant cells are just beginning to appear in the lungs, some of these myeloblastic cells will be found. In the kitten of 1 or 2 months, when the number of giant cells in the lungs is noticeably increased, the myeloblastic cells are conspicuous on account of their number and their apparent activity, as indicated by their large size and varying shapes. The histological picture indicates a relationship between these cells and the megacaryocytes and tends to support the theory that the giant cells develop from them in the lungs after birth, as they do in the liver before birth.

Megacaryocytes of the Bone Marrow and Other Tissues

Most of the authors who have studied the subject (Bunting, Ogata, Di Guglielmo, Kuczynski, Cesaris-Demel, Firket, Bianchini, Downey, Minot, Sabin, *et al.*) have accepted the theory of Wright that the blood platelets are produced from the cytoplasm of the megacaryocytes, and that in the adult animal this process takes place in the bone marrow. Sections of the marrow of the cat (femur), preserved and stained by the methods described, give excellent examples of the appearances described by Wright. The megacaryocytes are present in numbers, both in the immature and the mature form. In the latter

the cytoplasm is filled with granules that stain like those in the blood platelets, and frequently the cytoplasm sends out processes or pseudopodia which at times penetrate into the blood channels. The picture as a whole can scarcely be given any other interpretation than that proposed by Wright. There are, however, some known facts which, on the surface, at least, seem to be at variance with this interpretation, so far as the marrow is concerned. In sections or smears of the marrow few platelets are found. They are noticeably less numerous than in the lungs or spleen, and are fewer in number than would be expected on the theory that they are being constantly produced by the marrow megacaryocytes. Cramer and Drew have commented upon this fact, and it will be remembered that in our perfusion experiments which included the bone marrow, as in those of Drinker and Drinker in which the marrow alone was perfused, the effluent was very deficient in platelets. There was no indication of a supply of these elements in the marrow comparable to that found for the lungs. Clinical and experimental data reported from various sources indicate a lack of correlation between platelets and marrow megacaryocytes. The platelets may be reduced greatly in number or entirely eliminated with no obvious change in the number of megacaryocytes, or the marrow may show no megacaryocytes while the blood still contains platelets (Bianchini, Barbieri, Filo, Lawrence and Mahoney, Stebbins and Carns, Willi). Observations of this kind indicate the existence of some unknown factors bearing upon the relationship of the megacaryocytes of the marrow to the blood platelets. The participation of the lungs in the process of platelet production is one such factor that has not been considered heretofore.

On the histological side alone the connection of the mature form of megacaryocyte with platelet formation seems so convincing that one may assume that wherever such cells occur they are capable of discharging this function. As is well known megacaryocytes are found in several organs, and in the course of development of the fetus a certain sequence is observed. They appear first in the liver and subsequently in the spleen and marrow. We have been able to follow this march in the case of the cat.

In a fetus of about 80 mm. they are just beginning to appear in the marrow of the long bones. They are few in number and the cytoplasm is as yet immature, in

that platelet granules are not present. In the liver and the spleen, however, fully developed megacaryocytes are found, those of the liver giving evidence of greater activity, as indicated by the number that contain fully matured platelet granules in the cytoplasm. The lungs at this age contain no giant cells. In the circulation blood platelets are present although few in number. If the theory that they are formed by megacaryocytes is sound, then at this time only the giant cells of the liver and spleen take part in the process. In an older embryo, 115 mm., not far from term, the megacaryocytes in the liver and spleen were distinctly more numerous and the presence of processes of the cytoplasm indicated a greater degree of activity, but the maximum focus of activity still appeared to be in the liver. The number of platelets in the blood was increased, reaching a figure of 55,000 per c.mm. for a red cell count of 4,500,000. In the marrow of the femur the number of giant cells was distinctly greater than in the fetus of 80 mm. as determined from longitudinal sections through the entire bone. Toward the middle of the shaft some were found in which platelet granules were present in the cytoplasm.

At birth and shortly afterward the liver still contains megacaryocytes, but some of them show signs of degeneration in the nucleus, and the seat of activity has quite evidently been transferred to the spleen and the marrow. In the lungs at this time megacaryocytes begin to appear, a few being found in each section. Finally in an examination made of a cat approximately 2 months old the giant cells of the marrow were numerous and resembled those of the adult in variety and in evidences of activity, and in the lungs their number was distinctly larger than in the newly born animal. In the spleen, on the contrary, no megacaryocytes could be found.

In general, it may be said, for the cat, that several months after birth and in the adult animal the megacaryocytes are concentrated chiefly in the marrow and the lungs. In the marrow the histological picture indicates active platelet production, but this inference is not supported satisfactorily by the experimental results obtained from perfusions of the marrow and from actual platelet counts in the venous blood returning from the marrow. In the lungs, on the other hand, the histological evidence for active platelet formation by the giant cells is confirmed by the experimental results described in this paper, and one is justified in believing that the lungs constitute the main source of platelet production.

The functional relationship between the megacaryocytes of the lungs and those of the marrow is uncertain. Three possibilities may be considered. First, the megacaryocytes of the lungs may be explained, in accordance with the usual view, as migrants or emboli from the marrow which have been carried off in the blood stream and

caught mechanically in the lungs where they remain until degeneration and disintegration occur. We have given reasons for believing that this view does not fit the facts in the case. Second, it may be supposed that the megacaryocytes of the marrow after reaching a certain degree of maturity migrate to the lungs where, under different and presumably more favorable environmental conditions, they enter into full activity. This view assumes a mechanism of adjustment or accommodation for which there is no evidence and which seems to be intrinsically improbable, and it implies also a more or less constant transfer of megacaryocytes from marrow to lungs which is not borne out by the results of blood examinations. The third view, which seems to us the most probable, is that the lung megacaryocytes develop in the lungs from myeloblastic cells in the same manner that they do in the liver, spleen, and marrow. The reasons for the transfer of megacaryocytic activity from liver to spleen, and from spleen to marrow in the early stages of life are not apparent, but there seems to be no question that it takes place. In the same sense we may believe that in extrauterine life the conditions favor the development of these cells in the marrow and the lungs, and the experimental facts indicate that the place of greatest functional activity is in the lungs. On the histological evidence, however, one must admit that the megacaryocyte wherever found has the function or the potential function of producing platelets from its cytoplasm. It constitutes, so to speak, a unicellular gland which gives off a solid secretion in the form of platelets. The eventual solution or disintegration of the platelets presumably adds to the plasma important constituents the nature and significance of which are at present not definitely known.

SUMMARY

1. A new fixing solution is described, which preserves the platelets and prevents contact hemolysis of the erythrocytes, so that counts of both corpuscles may be made in the same preparation.
2. Comparative counts of platelets in arteries and veins show that arterial blood contains a larger number of platelets than venous blood. This difference is accentuated under experimental conditions that cause a reduction in the number of platelets. It is concluded that new platelets are added to the blood in the capillary areas of the lungs,

and that there is a corresponding destruction of platelets as the blood passes through the capillary areas of the systemic circulation.

3. Perfusion of the lungs with a platelet-preserving solution, compared with that of other organs, gives evidence of the existence of a source of platelet material in the lungs.

4. Histological examination of the lungs with a technique adequate to give a differential staining of platelet material demonstrates the presence of giant cells in the lungs, and supports the view that they are active in the production of platelets.

5. In extrauterine life giant cells are concentrated in the marrow and the lungs, with the maximum of their activity in platelet production in the lungs.

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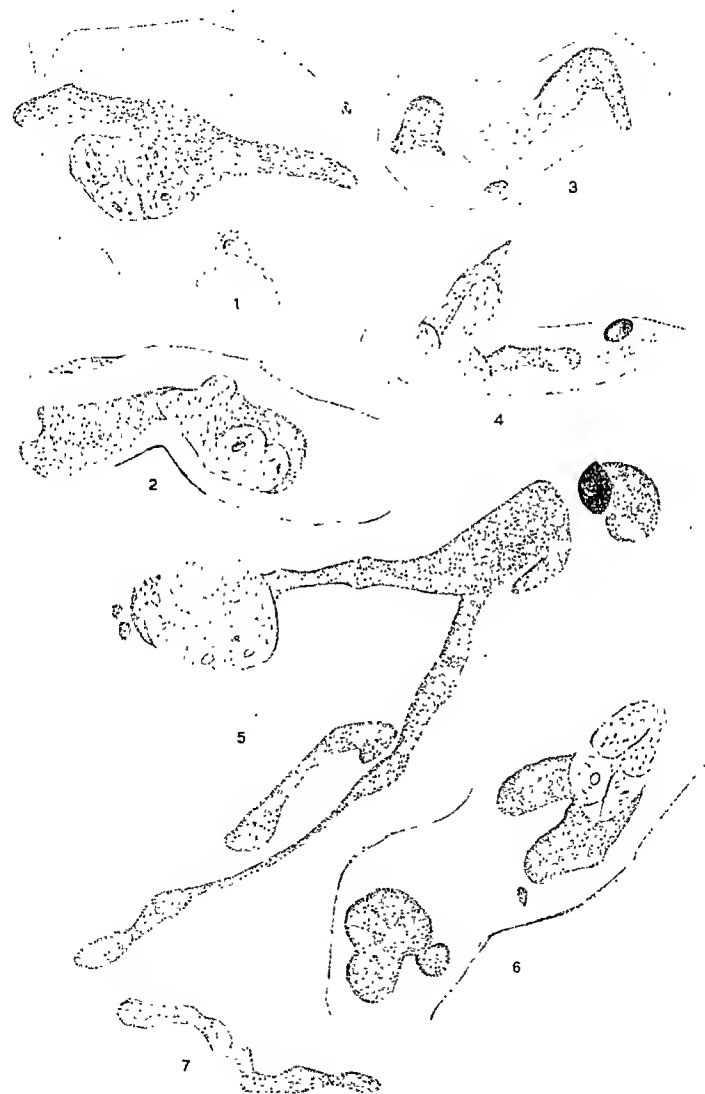
EXPLANATION OF PLATE 6

Megacaryocytes from the lungs of the cat, showing the character of the platelet processes. From camera lucida drawings.

FIGS. 1, 2, 3, 4, and 5. From the lung of a cat 5 days after partial defibrination of the blood.

FIG. 6. From the lung of a normal animal.

FIG. 7. An example of the detached platelet processes lying free in the capillaries—specimen from lung of normal animal.



THE EFFECTS OF GONADOTROPIC HORMONES IN THE TREATMENT OF EXPERIMENTAL TUBERCULOSIS*

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Whether the influence of pregnancy on the course of pulmonary tuberculosis is beneficent or malign is a problem which has baffled many clinicians and still remains unsettled. The early clinicians noted improvement during gestation and even recommended pregnancy as a therapeutic for tuberculous girls. However, at the beginning of this century the prevalent attitude was that pregnancy was very much to be avoided in the tuberculous woman. In recent years, this point of view has again been considerably modified. Although certain authors report aggravation of the tuberculous process during pregnancy in a percentage of instances varying anywhere from 10 to 100 per cent (1-3), a strong body of evidence is accumulating that pregnancy has no harmful effect in tuberculous women providing the disease is not too advanced or active and progressive (4-8).

It is apparent that conflicting points of view still prevail when pregnancy is considered together with its sequelae, parturition and puerperium. A much greater unanimity of opinion is encountered when the period of gestation is considered apart from the severe incident of delivery. Most observers find an amelioration of tuberculous disease during the course of gestation. Even those authors who hold that pregnancy is to be avoided in the tuberculous woman admit an improvement of the tuberculous condition during the early months of pregnancy. For example, Floyd (9) speaks of "a seeming improvement or arrest of the tuberculous process frequently noticed after the first months of pregnancy." Robinson (10) concludes that although parturition involves a special risk for the phthisical woman, the patient may show an apparent improvement during pregnancy; this beneficial effect usually disappears in later pregnancy.

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The explanations that have been offered for the amelioration of symptoms in the tuberculous during pregnancy have been manifold. Sabourin (11) stresses the absence of catamenial intoxication; Ingraham (12), the care and rest that the patient receives during pregnancy. Fishberg (13) considers the possible beneficial rôle of congestion of the respiratory mucous membranes. Brachman (14) believes that the elevation of the diaphragm during gestation puts the lung at partial rest.

On the other hand, various theories have also been advanced in explanation of the possible harmful effect of pregnancy on tuberculosis. Jameson (15) regards the alteration of capillary permeability incident to the gravid state as a possible cause. Metabolic changes of pregnancy that have been considered in this connection include hypercholesterolemia (16), demineralization, and increased basal metabolism (15).

Studies in the experimental animal have not led to any definite conclusions. A favorable effect of pregnancy on experimental tuberculosis in guinea pigs has been reported by Müller (17), Jameson (15), Burke (18), and Bogen (19). However, Herrmann and Hartl (20) came to a contrary conclusion. Schmidt-Lange (21) criticizes the work of Herrmann and Hartl, and in an extensive series of experiments, he was unable to determine an unfavorable effect of pregnancy on the course of tuberculosis in guinea pigs.

A phase of the problem which appears to have been inadequately considered is the change in endocrine balance which is such a prominent feature of pregnancy (*cf.* Krause (22)). Presumptive evidence for increased hormone secretion exists in the characteristic hypertrophy which occurs in the pituitary, thyroid, and adrenal cortex. There is also evidence of hyperfunction of the parathyroid and pancreas. The placenta contributes its quota of hormones. However, exact quantitative estimates indicating increased hormone secretion have been obtained only for the estrogenic and gonadotropic hormones. The appearance of the gonadotropic hormone in the urine is so characteristic of human pregnancy that it is universally used as a diagnostic test for gestation (Ascheim-Zondek test (23)).

We undertook to investigate the effect of the gonadotropic hormone on experimental tuberculosis, in order to determine whether this hormone might be a factor in the increased resistance displayed by pregnant women.

In part of our experiments we employed blood serum from a 4 months pregnant mare. The serum (Squibb) was first titrated for gonadotropic hormone, by injecting small amounts into mice, and found to contain more than 1300 mouse

units per liter. Such serum, of course, contains all the hormones characteristic of pregnancy, especially the estrogenic follicular hormone, theelin, which increases throughout the course of gestation. We also studied the effect of the isolated gonadotropic hormone. For this purpose we utilized two proprietary preparations of this substance, antuitrin-S (Parke, Davis) and follutein (Squibb), both obtained from human pregnancy urine. Glandular anterior pituitary extract (Squibb), which contains growth-promoting, thyrotropic, and gonadotropic principles, was also included in this study, as was the placental extract, emmenin (Ayerst, McKenna and Harrison).¹

After completion of the experiments here reported, we learned of certain Italian publications on related studies. Addressi (24) injected whole urine of pregnant women in tuberculous rabbits and found that the tuberculous process was accelerated. Of course, the repeated injection of whole urine introduces many factors in addition to the pregnancy hormones. Vercesi and Merenda (25) used isolated preparations of the gonadotropic and estrogenic hormones and concluded that these hormones do not aggravate tuberculosis in guinea pigs. However, only 5 animals were treated with the gonadotropic hormone and the infecting dose of tubercle bacilli was so great as to cause rapid generalization of disease and death within an average of 22 days. Repetti (26) could not detect any effect of the estrogenic hormone, folliculin, on tuberculosis in guinea pigs.

EXPERIMENTAL PROCEDURE

Rabbits and guinea pigs were the experimental animals employed, and only those which did not react to tuberculin were used. Weekly weight records were obtained throughout the course of the experiments. However, no useful correlation could be made between the weight curves and extent of disease. All animals were injected subcutaneously in the groin with a weighed amount of bovine tubercle bacilli (B1) in saline suspension. With the exception of the first experiment, hormone administration was begun the day of infection and was continued daily, omitting Sundays. In each experiment a group of infected animals remained untreated to serve as controls. At various intervals after infection one animal from each group was sacrificed and a comparative study was made of the extent of macroscopic tuberculosis. The estimate of the severity of the infection was based on the number, nature, and distribution of lesions in the susceptible organs, and was expressed in a scale of from 0 to 4+. The system of grading was essentially the same as employed by Petroff and Steenken (27): 0

¹ We are greatly indebted for the generous supply of hormones to Parke, Davis and Co., E. R. Squibb and Sons, and Ayerst, McKenna and Harrison.

indicates no tuberculous involvement; 1+ indicates a very few scattered tubercles in one or two of the susceptible organs; 2+ indicates a moderate number of tubercles in one or more organs; 3+ indicates somewhat generalized distribution in all organs or, in some instances, extensive active disease in one organ; 4+ indicates severe progressive generalized tuberculosis. Sections of the spleen, lymph glands, liver, kidneys, and lungs were fixed in Zenker's solution and studied microscopically. The histopathology of lesions in the treated animals did not appear to vary from that of controls.

The Effect of Antuitrin-S, Follutein, and Anterior Pituitary Extract on Experimental Tuberculosis in Rabbits

In our first experiment we utilized 24 young female rabbits, divided into 4 groups of 6 animals each. One group received no hormone treatment and served as controls. The remaining 3 groups received subcutaneous injections of one of the following hormones daily, except Sundays: (a) antuitrin-S, 0.75 cc., or 75 rat units; (b) follutein, 0.3 cc., or 75 rat units; (c) anterior pituitary extract, 2.0 cc., or 20 rat growth units. Antuitrin-S and follutein are both standardized according to their content of gonadotropic hormone, i.e., ability to produce mature follicles, hemorrhagic follicles, and corpora lutea in ovaries of immature rats. Anterior pituitary extract is standardized according to its growth-promoting principle. The three experimental groups were pretreated for 10 days with the stated dose of hormone, and together with the control group were then infected subcutaneously with 0.01 mg. of a pathogenic bovine strain of tubercle bacillus (B1). Hormone treatment was continued until the animals were autopsied, at 74 to 114 days after infection. In general, when an animal died in one group, an animal from each of the other groups was also killed for comparative study. In each animal, the involvement of the susceptible organs was recorded on a 0 to 4+ scale, and a general estimate of the extent of the disease in the animal was obtained according to the system outlined above; and, finally, an average for the tuberculous involvement of each group as a whole was calculated (Table I).

It soon became evident that the animals of this experiment which were treated with the gonadotropic principle developed distinctly less tuberculosis than had the anterior pituitary-treated group and the controls. In every instance these animals showed less disease than those with a comparable survival period in the latter 2 groups. No tuberculosis whatsoever was found in the liver, spleen, or lymph glands of any animal treated with gonadotropic hormones. As the experiment progressed, it was apparent that the antuitrin-S-treated rabbits were slightly less diseased than the follutein-treated animals. One rabbit in the antuitrin-S group showed no tuberculosis in any

organ, although it survived for 92 days after infection. In another, which lived for 74 days, the infection in the lung was discernible only by microscopic examination. In the group which survived for 107 days, the antuitrin-S-treated animal showed only 1+ tuberculosis in the lungs, while the corresponding anterior pituitary-treated animal showed not only the involvement noted in the table, but also disease in the omentum, peritoneum, and pleura. The corresponding

TABLE I
Effect of Various Hormones on Tuberculosis in Female Rabbits

Time infected days	Antuitrin-S 0.75 cc.					Follutein 0.3 cc.					Anterior pituitary 2.0 cc.					No hormone								
	Lung	Kidney	Liver	Spleen	Lymph glands	General estimate	Lung	Kidney	Liver	Spleen	Lymph glands	General estimate	Lung	Kidney	Liver	Spleen	Lymph glands	General estimate						
74-80	1	0	0	0	0	1+	1	0	0	0	0	1+	4	0	4	4	0	4+	4	0	0	0	0	3+
90	2	2	0	0	0	2+	2	0	0	0	0	2+			†				2	4	4	4	0	4+
91-93	0	0	0	0	0	0*	2	2	0	0	0	2+	4	0	0	0	0	3+	2	0	1	1	0	3+
104	1	0	0	0	0	1+	0	1	0	0	0	1+	4	0	4	4	0	4+	4	1	1	0	0	4+
107	1	0	0	0	0	1+	4	3	0	0	0	4+	4	4	0	0	4	4+	4	4	4	2	4	4+
114	3	1	0	0	0	3+	3	0	0	0	0	2+	4	1	4	1	0	4+	4	4	4	4	0	4+
Average tuberculous involvement.						1.3+	2+						3.8+						3.7+					

Degree of tuberculous involvement recorded as 0 to 4+.

Rabbits injected subcutaneously with 0.01 mg. B1. Hormone treatment begun 10 days before infection and continued daily except Sundays.

* Indicates animal died spontaneously; death was usually due to intercurrent infection.

† This animal died 27 days after infection as a result of injury to spine, hence is not included in table.

control had tuberculosis of the diaphragm and pericardium, as well as in the more susceptible organs.

The average degree of tuberculous involvement of the various groups is recorded as: antuitrin-S 1.3+, follutein 2+, anterior pituitary extract 3.8+, and controls 3.7+. Of course these average values cannot be taken to indicate actual quantitative ratios for the different groups since they are based upon a series of relative, not

absolute numbers. Nevertheless they may serve to give some idea of the comparative involvement of each group as a whole. The conclusion that the gonadotropic hormones have retarded the progress of disease is borne out by the consideration that in every one of the six sets of comparative autopsies, the animals treated with the gonadotropic hormones showed less disease than did the corresponding control animals.

The Effect of Antuitrin-S, Follutein, and Anterior Pituitary Extract on Experimental Tuberculosis in Male Guinea Pigs

In this experiment the same three hormone preparations were tested on guinea pigs instead of on rabbits. Male animals were employed for this experiment as a convenient way of avoiding the possible complication of pregnancy. The gonadotropic hormones employed are known to affect the gonads of the male as well as the female. Anterior pituitary extract (growth hormone) also is effective in both male and female animals. We used 24 albino male guinea pigs, weighing 450 to 500 gm., none of which reacted to tuberculin. All the animals were infected with 0.001 mg. B1 (bovine tubercle bacilli), subcutaneously. They were then divided into 4 groups of 6 each. The first group was treated with antuitrin-S, 0.4 cc., or 40 rat units; the second with follutein, 0.16 cc., or 40 rat units; the third with anterior pituitary extract, 1.0 cc., or 10 rat growth units; while the fourth group was kept untreated for control purpose. The dosage was half that used in the rabbit experiment, but, considering the difference in weight of the animals, was proportionately larger. Hormone treatment was begun on the day of infection, and was continued daily except Sundays. The animals were sacrificed in groups of 4, at 35, 36, 37, 38, 39, and 41 days after infection. We chose a short survival period since it is known that the administration of hormones has a maximum stimulating effect for only a few weeks. It is believed that the administration of hormones over a long period of time may even have a harmful effect on the glands of internal secretion, due to overstimulation followed by a period of depression (Zondek (28)), or possibly because of the production of antihormones (Collip and Anderson (29)).

Again, in this experiment, the gonadotropic principle gave the best results, that is to say, animals treated with antuitrin-S and follutein had less tuberculosis than those treated with anterior pituitary extract and non-treated controls. Antuitrin-S again seemed much more effective than follutein in inhibiting the progress of the disease. In every instance, the antuitrin-S-treated animal showed considerably less disease than the corresponding animals in the other groups. The antuitrin-S-treated animal which survived for 37 days showed no

tuberculosis on gross examination, and microscopic study revealed only a slight involvement of one lymph gland. 5 of the 6 guinea pigs treated with this hormone were recorded as 1+ in the general estimate of disease, in striking contrast to the more or less generalized tuberculosis found in the other groups of animals. It is interesting to note that in this experiment the anterior pituitary-treated animals showed as much disease as the controls, and in some instances distinctly more. The values for average involvement were: antuitrin-S 1.2+, follutein 3.2+, anterior pituitary extract 4+, controls 3.8+ (Table II).

The Effect of Antuitrin-S and Anterior Pituitary Extract on Experimental Tuberculosis in Male Guinea Pigs

In order to confirm our results, we repeated our second experiment with certain modifications. We used a smaller infecting dose, 0.0001 mg. of hovine tubercle bacilli (B1) injected by the same subcutaneous route. The hormones were administered as before except that follutein was not used, as this preparation had previously proved less effective than antuitrin-S.

Twelve male guinea pigs, weighing from 500 to 700 gm., all non-tuberculin reactors, were infected. They were then divided into 3 groups of 4. The first group was treated with antuitrin-S, 0.4 cc., or 40 rat units, and the second with anterior pituitary extract, 1 cc., or 10 rat growth units; while the third group served as controls. In each group, one animal was sacrificed at 35 days after infection, one at 39, one at 40, and one at 41.

The efficacy of antuitrin-S was again strikingly demonstrated. The antuitrin-S-treated animals were in every instance less severely involved than the corresponding controls. The average involvement of the antuitrin-S-treated group was only slightly more than half that of the anterior pituitary-treated animals, and less than half that of the controls (Table III). Of the individual animals, the only 2 with involvement estimated at 1+ appeared in the antuitrin-S-treated group. Noteworthy is the absence of lesions in the spleen and lymph glands of all 4 antuitrin-S-treated animals. The other 2 groups showed extensive involvement of these organs in most instances.

In an attempt to determine whether temporary hormone treatment would influence survival time, another group of 12 guinea pigs was infected and treated in exactly similar fashion. Treatment was dis-

TABLE II

Effect of Various Hormones on Tuberculosis in Male Guinea Pigs

Time infected	Antuitrin-S 0.4 cc.					Follutein 0.3 cc.					Anterior Pituitary 1.0 cc.					No hormone				
	Lung	Liver	Spleen	Lymph glands	General estimate	Lung	Liver	Spleen	Lymph glands	General estimate	Lung	Liver	Spleen	Lymph glands	General estimate	Lung	Liver	Spleen	Lymph glands	General estimate
days																				
35	1	1	0	0	1+	0	2	0	0	2+	4	4	4	4	4+	4	4	4	0	4+
36	0	1	0	0	1+	4	1	4	0	1+	2	4	4	0	4+	4	4	4	0	4+
37	0	0	0	2	1+	3	4	4	4	4+	4	4	4	4	4+	4	4	4	0	4+
38	0	2	1	1	2+	3	1	2	1	3+	4	4	4	1	4+	4	4	4	4	4+
39	0	1	0	0	1+	3	2	2	0	3+	4	4	4	3	4+	4	2	4	4	4+
41	0	1	0	0	1+	3	4	2	1	3+	4	4	4	0	4+	2	4	3	0	3+
Average tuberculous involvement.....					1.2+															

Degree of tuberculous involvement recorded as 0 to 4+.

Guinea pigs injected subcutaneously with 0.001 mg. B1. Hormone treatment begun day of infection and continued daily except Sundays.

TABLE III

Effect of Various Hormones on Tuberculosis in Male Guinea Pigs

Time infected	Antuitrin-S 0.4 cc.					Anterior pituitary 1.0 cc.					No hormone				
	Lung	Liver	Spleen	Lymph glands	General estimate	Lung	Liver	Spleen	Lymph glands	General estimate	Lung	Liver	Spleen	Lymph glands	General estimate
days															
35	0	1	0	0	1+	0	4	0	0	2+	4	4	2	0	4+
39	0	1	0	0	1+	4	4	3	4	4+	4	3	0	0	3+
40	4	1	0	0	3+	4	3	3	4	4+	4	4	4	4	4+
41	1	1	0	0	2+	1	4	3	2	3+	4	1	4	4	4+
Average tuberculous involvement.....					1.8+										

Degree of tuberculous involvement recorded as 0 to 4+.

Guinea pigs injected subcutaneously with 0.0001 mg. B1. Hormone treatment begun day of infection and continued daily except Sundays.

continued at 41 days after infection, and the animals survived from 149 to 305 days. It appeared that hormone treatment did not lengthen survival time nor lessen eventual involvement, but one is not justified in drawing conclusions from such a small group of animals, many of which died of intercurrent infection.

The Effect of Antuitrin-S, Pregnant Mare Serum, and Emmenin on Experimental Tuberculosis in Female Guinea Pigs

The results with the gonadotropic hormones were considered of such significance as to warrant a more comprehensive study of the hormone factors of pregnancy in relation to resistance to tuberculosis. We decided to use serum from a pregnant mare, since such serum contains all the hormones of pregnancy, and probably in their optimal proportions. We thought that even more favorable results might be obtained with such serum than with the gonadotropic principle alone. As previously mentioned, the serum we employed was obtained when the mare was 4 months pregnant. At this time the serum is rich in gonadotropic principle, estrogenic principle, and other hormones of pregnancy. We also included in this experiment another hormone characteristic of pregnancy, namely, the placental hormone, emmenin. This hormone is distinctive in that it is very successfully given *per os*.

In this experiment female guinea pigs were employed. It was thought that the administration of these hormones to female animals might provide a closer analogy to the situation in the pregnant woman. Proper precautions were taken to exclude pregnant animals from this experiment. 35 female guinea pigs, weighing 400 to 550 gm., were injected subcutaneously with 0.001 mg. of bovine tubercle bacilli (B1). They were then divided into 5 groups, as follows: the first 2 groups served as controls, one remaining untreated and the second receiving normal mare serum daily to serve as controls for the animals receiving pregnant mare serum; the third group received antuitrin-S, 0.4 cc., or 40 rat units; the fourth was treated with pregnant mare serum, 0.5 cc., or 0.7 mouse unit; and the fifth was treated with emmenin, 0.5 cc., or 5 oral day units. The hormones were administered daily except Sundays, by subcutaneous injection except in the case of emmenin, which was given twice daily *per os*. One animal from each group was killed at 28, 32, 35, 36, 37, 38, and 39 days after infection.

The value of gonadotropic hormone treatment was again apparent. In 5 of 7 instances the antuitrin-S-treated animal showed distinctly

TABLE IV
Effect of Various Hormones on Tuberculosis in Female Guinea Pigs

Time infected days	Antultrin-S 0.4 cc.					Pregmant mare serum 0.5 cc.					Emmenin 1.0 cc.					Normal mare serum 0.5 cc.					No hormone				
	Lungs	Liver	Spleen	Lymph glands	General estimate	Lungs	Liver	Spleen	Lymph glands	General estimate	Lungs	Liver	Spleen	Lymph glands	General estimate	Lungs	Liver	Spleen	Lymph glands	General estimate	Lungs	Liver	Spleen	Lymph glands	General estimate
28	0	1	1	2	1+	0	1	0	1	1+	0	4	2	4	4+	3	4	4	4	4+	3	4	3	3	4+
32	1	2	4	1	3+	2	1	2	2	2+	3	4	4	4	4+	1	3	1	3	3+	2	2	4	4	3+
35	0	1	1	2	1+	1	0	3	3	3+	0	2	1	3	3+	1	4	3	3	3+	1	4	4	4	3+
36	0	1	1	2	1+	1	3	3	3	3+	1	2	4	4	4+	3	4	2	3	2+	1	2	4	4	3+
37	1	3	2	2	2+	0	1	1	3	1+	4	4	4	4	4+	3	4	1	3	3+	3	3	4	4	3+
38	0	0	1	3	1+	0	2	3	2	2+	1	3	3	3	3+	2	3	3	3	3+	2	2	3	4	3+
39	2	3	3	3	3+	1	1	1	1	1+	2	3	4	4	4+	1	2	2	4	3+	3	2	3	2	3+
Average tuberculous involvement.....					1.7+					1.9+					3.7+					3+					3.4+

Degree of tuberculous involvement recorded as 0 to 4+.

Guinea pigs injected with 0.001 mg. bovine tubercle bacilli (B1), subcutaneously. Hormone treatment begun on day of infection and continued daily except Sundays.

* Animal died of intercurrent infection.

less tuberculosis than did the corresponding control. In the other 2 instances the animals of both groups were equally involved. The pregnant mare serum gave approximately similar results. In 6 instances the animals treated with pregnant serum manifested less disease than the corresponding untreated animals and, in 5 instances, less than the guinea pigs treated with normal serum. The average degree of involvement was 1.7+ for the antuitrin-S group and 1.9+ for the pregnant serum group, values significantly lower than for all other groups. When considered from the point of view of animals showing minimal (1+) tuberculosis, these same 2 groups are again outstanding—the number for each group being: antuitrin-S 4, pregnant mare serum 3, all other groups 0 (Table IV).

Daily injection of normal mare serum did not result in any appreciable lessening of disease, although in 3 instances the treated animal showed slightly less involvement than the non-treated control. The average value is therefore slightly lower than for the non-treated controls.

The emmenin-treated guinea pigs fared worst of all the groups. In comparison to the non-treated controls the animals in this group showed more extensive disease in 3 instances, and less disease in only one instance. The average value for the group as a whole indicates somewhat more severe involvement than the controls.

DISCUSSION

It is apparent from these experiments that the gonadotropic hormone obtained from the urine of pregnant women exerts a favorable influence on the progress of experimental tuberculosis in both male and female animals. Of all the animals treated with antuitrin-S (6 rabbits and 17 guinea pigs), only 2 guinea pigs failed to show less tuberculosis than did the corresponding controls. Follutein, which contains the same hormone, also proved of some value in retarding the progress of disease, but was not as effective as antuitrin-S. It appears possible that the poorer results obtained with follutein may be due to the high concentration of glycerol present in this preparation. Long and Vorwald (30) have shown that injection of glycerol enhances the multiplication of tubercle bacilli in rats.

Pregnant mare serum, which is a rich source of gonadotropic

hormone, also proved very efficacious in retarding tuberculosis. In the one series in which it was tested, it gave results very nearly as good as did antuitrin-S. But it may be added that this serum which contains all the hormones of pregnancy did not prove superior to the use of the gonadotropic principle alone.

Anterior pituitary extract, containing growth, sex, and thyrotropic principles, proved of no value in retarding the progress of the disease, and in many instances animals treated with this hormone showed more tuberculosis than did the controls. One is reminded here of the lowered resistance to tuberculosis displayed by man during the period of most active growth—presumably the time when the secretion of growth hormone is greatest.

Placental extract (emmenin) given by mouth was likewise entirely ineffective in preventing progress of the disease, which in some instances was more invasive in the treated animal than in the control.

The results obtained suggest that the gonadotropic principle which appears during pregnancy may be an important factor in the increased resistance displayed by the pregnant tuberculous woman. The breakdown of this resistance that occurs during the later stages of pregnancy may perhaps be correlated with the diminution of this hormone, which begins after about the 5th or 6th month and ends with the entire disappearance of the hormone a few days after delivery.

The mechanism whereby the gonadotropic hormone influences tuberculosis remains to be determined. Experiments are planned in castrated rabbits to decide whether the hormone acts necessarily through a stimulation of the sex glands.

SUMMARY

Experimental tuberculosis in rabbits and guinea pigs was favorably influenced by the administration of antuitrin-S, pregnant mare serum, and, to a lesser extent, follutein. No retardation of disease was obtained by the use of either anterior pituitary extract or emmenin.

The results suggest that the gonadotropic hormone may be a factor in the temporary amelioration of symptoms observed in tuberculous women during pregnancy.

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IMMUNIZATION OF RABBITS TO INFECTIOUS PAPILLOMATOSIS

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Rabbits in which papillomatosis has developed following infection with papilloma virus are partially or completely resistant to reinfection. When areas of normal skin on such papilloma-bearing animals are inoculated with virus by the usual methods, the new sites of inoculation either fail completely to develop papillomas or develop them after unusually long incubation periods in smaller numbers than do areas on corresponding control animals (1). In addition, sera from papillomatous rabbits contain antibodies capable of neutralizing the virus (1), and the experiments of Kidd, Beard, and Rous (2) indicate that the height of antibody titer attained may be, in many instances, directly dependent upon the amount of papillomatous tissue developed following infection. In all of these experiments the development of resistance to infection with the virus, or of virus-neutralizing antibodies, was associated with the formation of papillomas on the rabbits studied. It was of interest in several directions, as will become evident later, to determine whether rabbits could be immunized to papillomatosis by means other than actual infection of the skin with the virus.

Earlier work had indicated that the virus is strictly dermatotropic in its tissue affinities and induces papillomatosis only when inoculated intradermally or applied to the scarified skin, or when it localizes in the damaged epidermis after injection into the blood stream. Introduced intravenously, intraperitoneally, or intracerebrally into an animal with normal skin, the virus causes neither clinically recognizable illness nor visceral pathology discernible at autopsy (1).

Some of the rabbits used in determining the dermatropism of the

virus were later tested for resistance to virus applied to the scarified skin. 5 that had been inoculated intracerebrally and 2 that had been inoculated intravenously with active Berkefeld filtered virus, and that had failed to develop detectable papillomas following these inoculations, were later found to be partially or completely resistant to infection with virus applied to their scarified skin. This finding suggested that multiple injections of papilloma virus by routes other than those resulting in infection might prove an efficient immunizing procedure.

EXPERIMENTAL

Preparation of Papilloma Suspensions.—Papillomatous tissue that had been stored in 50 per cent glycerol in the refrigerator for 2 months or longer was rinsed in three changes of physiological saline to remove adherent glycerol. Weighed amounts were then ground to a fine paste with sterile sand in a mortar, and physiological saline was slowly added, with constant grinding, to make a 5 per cent final suspension. The suspensions were either allowed to sediment in tall tubes or were centrifuged, and the supernatant fluid, which was free of gross particles and only moderately turbid, was removed by pipette and saved. In the tables, as well as elsewhere in this paper, the supernatant fluids thus obtained will be referred to as 5 per cent suspensions. Suspensions to be used for immunization or for infection were prepared in identical manner, except that the latter were usually 0.5 per cent instead of 5 per cent. The prolonged period of storage of the papillomatous tissue in glycerol prior to its use served the double purpose of diminishing the number of bacterial contaminants and of insuring against the survival of any viable epithelial cells which might possibly influence the outcome of immunization experiments.

Immunization of Domestic Rabbits

1. By the Administration of Cottontail Rabbit Papilloma Virus Intraperitoneally or Intravenously.—Each of 3 domestic rabbits was injected intravenously with 2 cc. of the supernatant fluid from a centrifuged 5 per cent suspension of glycerolated cottontail rabbit papillomas. Three other rabbits received the same amount of suspension intraperitoneally. The injections were repeated 8 days later. A fresh sterile needle, applied to the syringe after loading, was used for each animal to minimize possible skin epithelium infection at the site of injection. Despite this precaution, 1 of the rabbits injected intraperitoneally developed papillomas at the site of needle puncture. Furthermore, another, injected intravenously, developed scattered papillomas over its shoulders, neck, and back, obviously where the virus had localized in damaged skin. In the remaining 4 rabbits, repeated and thorough palpation of the skin over the entire animal failed to reveal the presence of papillomas. The papilloma suspension used to vaccinate caused diffuse papillomatosis when applied to the scarified skin of a control rabbit, showing that it was rich in virus.

The 6 vaccinated rabbits were tested for immunity 20 days after their second intravenous or intraperitoneal injection. The shaved skin of their abdomens was scarified with sandpaper over an area approximately 15 cm. square and a 5 per cent suspension of cottontail rabbit papilloma virus was applied as previously described (1). They were kept under observation for 6 weeks following infection. The results of the experiment are given in Table I.

Table I indicates that two intraperitoneal or intravenous injections of papilloma virus rendered rabbits partially or completely resistant to the pathogenic effect of the virus when applied to abraded skin. Partial resistance was evidenced by a prolongation in the period of

TABLE I
Immunization of Domestic Rabbits to Papillomatosis

Rabbit No.	Vaccination (2 injections of infectious 5 per cent suspension glycerolated papillomas)		Test for immunity (massive inoculation of scarified skin with suspension of infectious papillomas)	
	Route of injection	Papillomas resulting	Result	Incubation period
13-07	Intravenous	None	2 papillomas	<i>days</i> 29
13-14	"	Scattered on back	Negative	
13-15	"	None	"	
13-09	Intraperitoneal	"	9 papillomas	29
13-10	"	At site of needle puncture	2 "	21
13-13	"	None	10 "	29
13-25	Nil, control		Confluent papillo- matosis	7

incubation and by a great diminution in the number of papillomas developing in the area of skin inoculated. Papillomas appearing on the inoculated skin of the control rabbit were too abundant to count and very soon became confluent, producing a broad folded mass of keratinized papillomatous tissue. The resistance manifested by the 2 rabbits which developed papillomas following vaccination can obviously not be ascribed solely to the immunizing procedure since it is known (1) that papillomatous animals become partially or completely immune to reinfection. The resistance of the remaining 4 vaccinated rabbits was, however, clearly not the result of detectable infection of susceptible epithelium by the papilloma virus.

The inclusion of only a single control animal in experiments like that just described may, superficially, appear inadequate. However, the constant and predictable course of the disease and the knowledge that, in a series of infection experiments now large, no domestic rabbit naturally resistant to the virus has been encountered, made it unnecessary to use great numbers of control animals in the present experiments.

2. *By the Intraperitoneal Administration of Cottontail and Domestic Rabbit Papilloma Virus and by Non-Infectious Suspensions of Domestic Rabbit Papillomas.*—Work with the rabbit papilloma virus is complicated by the difficulty of transmitting it serially beyond the first passage in domestic rabbits. While virus is readily obtained from the naturally occurring papillomas of cottontail rabbits and from most of those experimentally produced in this species, it is rarely demonstrable in the papillomas it causes in domestic rabbits. In earlier work with the disease, active virus could not be recovered from the papillomas of any of a series of domestic rabbits tested (1). Later, when the question was restudied, it was obtained occasionally. Virus recovered from domestic rabbit papillomas sometimes proved further transmissible in domestic rabbits (3), and one strain has at present reached its 14th serial passage in this species.

It has yet to be determined why, in certain instances, domestic rabbit papillomatosis proves transmissible and in other instances non-transmissible, although it was suggested earlier that the inhibitory substance demonstrably present in many domestic rabbit papillomas (1) might render the virus non-infectious. But other possibilities exist and, among these, one of the most obvious is that many domestic rabbit papillomas may rapidly lose the virus responsible for their initiation and that, once cell proliferation is started, it continues without the virus stimulus. The likelihood that this is so in the papillomas that progress eventually to cancer has been eliminated by the experiments of Kidd, Beard, and Rous (4), in which they observed that rabbits bearing transplanted carcinomas, derived from papillomas, developed papilloma virus-neutralizing antibodies. They interpreted their findings to indicate that papilloma virus, though non-demonstrable by the usual infection test, was nevertheless present in the malignant metastases used for transplantation. The question

of whether or not virus is present in benign non-infectious papillomas has remained open. Yet another possibility to explain the usual non-transmissibility of infectious papillomatosis in domestic rabbits may be that the virus is, in this species, in some manner altered to a non-infectious phase. It seemed worth while to compare the immunizing ability of both infective and non-infective domestic rabbit papillomas with that of the highly infective cottontail rabbit growths in the hope that the data obtained might shed light on the question of the non-transmissibility of papillomatosis in domestic rabbits.

The experiment recorded in Table I had indicated that rabbits could be rendered resistant by virus given either intraperitoneally or intravenously. In the case of both routes, however, a danger existed that virus might infect susceptible epidermal cells, with a resulting formation of papillomas, perhaps multiple minute ones hidden in the fur, with resistance developing in consequence. Since this hazard seemed more easily controllable in rabbits injected intraperitoneally than in those injected intravenously, the intraperitoneal route was used in all the later experiments. The technique of inoculation was varied somewhat from that employed to begin with.

An area roughly 8 cm. square on the abdomen of each rabbit was shaved a day before the injection. This area was thoroughly moistened with alcohol at the time of intraperitoneal injection and a fresh needle, applied to the syringe after loading, was used for each animal. The needle was inserted at an angle through the skin so that it would traverse as much muscle and subcutaneous tissue as possible upon withdrawal and thus mechanically remove virus from its open tip before reaching susceptible epithelial cells at its point of skin penetration. After withdrawal of the needle, the puncture wound was daubed with an alcoholic solution of picric acid and its site marked with a skin pencil. 2 days after injection, 0.5 cm. of skin about the puncture wound was excised. Using these precautions, it has been possible to inoculate rabbits intraperitoneally with highly active virus without the development of papillomas at the site of skin puncture or, so far as could be told by repeated careful examination and palpation through the fur, elsewhere on the body.

In the present experiment each of a group of 8 domestic rabbits was injected intraperitoneally with 2 cc. of the supernatant fluid from a 5 per cent suspension of glycerolated cottontail rabbit papillomas. This suspension was known to be rich in virus and capable of producing confluent papillomas when applied to scarified rabbit skin. Each of 8 domestic rabbits in a second group was injected intraperitoneally with 2 cc. of the supernatant fluid from a 5 per cent suspension

of glycerolated domestic rabbit papillomas known to contain virus capable of infecting scarified rabbit skin (3). This virus had been transferred serially 12 times in domestic rabbits. Each of a third group of 8 domestic rabbits was injected intraperitoneally with 2 cc. of the supernatant fluid from a 5 per cent suspension of glycerolated domestic rabbit papillomas, proven by repeated test to be completely non-infectious when applied to scarified rabbit skin. This last suspension, so far as could be determined by the usual infection test, contained no papilloma virus. After an interval of 8 days, each of the injections was repeated, using the same precautions to prevent skin infection. The animals were then carefully observed for 20 days to note any papillomas that might develop either at the site of skin puncture or elsewhere on the body. None appeared and the animals were tested for resistance by inoculating their shaved, scarified abdominal skin over an area approximately 15 cm. square in the usual way (1) with 1 cc. of the supernatant of a 0.5 per cent suspension of glycerolated cottontail rabbit papillomas. Titration of this suspension in control rabbits revealed, as shown in Table II, that, diluted 1:1000 with physiological saline, it was still capable of producing scattered papillomas when applied to the scarified skin. From this, it is apparent that at least 1000 skin-infecting doses of papilloma virus were used in testing for immunity.

Sera of some of the vaccinated rabbits, drawn just before the test for immunity, were studied for their ability to neutralize papilloma virus. Control sera had been obtained from the same animals before vaccination. The usual virus neutralization technique was employed. The supernatant of a 5 per cent suspension of glycerolated cottontail rabbit papillomas, after filtration through paper (Whatman 42), served as virus and was mixed in equal parts with the dilution of serum to be tested. After storage overnight (17 hours) in the refrigerator, the mixtures were applied to the freshly scarified skin of domestic rabbits. Nine mixtures were tested on each rabbit. The nine inoculation sites, each 4 to 5 cm. across, were shaved a day before inoculation and thoroughly washed with tap water to insure a dry, soap-free skin surface for infection. Unshaved furry zones 1-2 cm. in width separated the shaved areas. Scarification was effected by means of a small piece of sterile sandpaper. 3 drops of the serum-virus mixture were then applied by pipette and immediately rubbed well into the scarifications with the rounded closed ends of sterile agglutination tubes. Inoculation of one area was completed before another was scarified. After inoculation the rabbit was held on its back for a few minutes until the abraded areas had partially dried. Bandaging, as described in the papilloma virus neutralization experiments of Kidd, Beard, and Rous (2), was not resorted to, but uninoculated control areas on some of the animals were scarified and no papillomas developed. Readings of the inoculated areas were made on the 12th and 26th days after inoculation. There was little change in the papillomas after this time, other than progressive enlargement.

The results of the experiments just outlined are given in Table II.

As shown in Table II, all 24 rabbits that received two intraperitoneal injections of suspensions of glycerolated papillomas were partially or completely resistant to infection with the virus of papillomatosis. The variation in resistance developed, as judged by the test infection, was probably dependent more upon differences in individual rabbits than upon differences in the suspensions used to vaccinate. It is known from earlier experiments (1) that only about one-half of the rabbits affected with papillomatosis are completely resistant to reinfection and it would be surprising if any artificial prophylactic measure produced an immunity greater than that conferred by actual infection of the skin by the virus. Papillomas developing in the partially resistant animals appeared only after unusually long incubation periods and were about the same numerically as those in control rabbits infected with 1:1000 dilutions of the test virus.

Judged by the test for active immunity, a suspension of non-infectious virus-induced domestic rabbit papillomas was as effective an immunizing agent as suspensions of papillomas, rich in virus, from either domestic or cottontail rabbits. Averaging the three groups in the experiment, 4 of the 8 rabbits in the first group vaccinated with non-infectious domestic rabbit papillomas were immune, while a total of 24 discrete papillomas appeared on the skin of the 4 partially immune animals. In the second group vaccinated with infectious domestic rabbit papillomas, 4 rabbits were immune and 23 papillomas appeared on the 4 partially immune animals. In the third group vaccinated with infectious cottontail rabbit papillomas, 3 were immune while 12 papillomas appeared on the 5 partially immune animals.

Judged on the basis of neutralizing antibodies in their sera, the rabbits vaccinated with cottontail material were slightly more immune than those receiving domestic rabbit papilloma suspensions. However, factors other than the amount of virus in the suspensions injected may have exerted an influence here. The group injected with cottontail material, for instance, received protein from a foreign animal species in addition to virus and this difference in inoculum, which could not be controlled in the other two groups, may have affected the antibody response to the virus. There was little, if any, difference between the groups injected with domestic rabbit papilloma suspensions; the virus-neutralizing antibody response of the rabbits receiving

TABLE II
Immunization of Domestic Rabbits to Papillomatosis by Means of Infectious and Non-Infectious Papilloma Suspensions

Rabbit No.	Vaccination (2 intraperitoneal injections 5 per cent suspension glycerolated papillomas)			Test for immunity (massive inoculation of scarified skin with suspension infectious papillomas)			Neutralization of papilloma virus by rabbit sera			
	Rabbit source	Infectious for skin	Papillomas resulting	Dilution 0.5 per cent papilloma suspension used	Result	Incubation period days	Virus + normal serum	Virus + serum after vaccination		
								Serum dilution		
								1:2	1:2	1:20
13-26	Domestic 12-86	No	None	Undiluted	Negative					
13-27	"	"	"	"	"	28				
13-28	"	"	"	"	10 papillomas	28				
13-29	"	"	"	"	7 "					
13-30	"	"	"	"	Negative					
14-04	"	"	"	"	6 papillomas	28	3+, 4+	0, 2+	0, 2+	4+, 4+
14-05	"	"	"	"	1 papilloma	28	3+, 4+	0, 0	0, 1+	0, 3+
14-06	"	"	"	"	Negative		4+, 4+	0, 0	0, 1+	0, 2+
13-31	12-70	Yes	"	"	2 papillomas	28				
13-32	"	"	"	"	Negative					
13-33	"	"	"	"	"					
13-34	"	"	"	"	"					
13-35	"	"	"	"	"					
14-07	"	"	"	"	7 papillomas	19	4+, 4+	0, 1+	0, 1+	2+, 3+
14-08	"	"	"	"	13 "	28	4+, 4+	0, 2+	3+, 4+	4+, 4+
14-09	"	"	"	"	1 papilloma	32	3+, 4+	0, 1+	3+, 4+	2+, 4+
13-36	Cottontail 12-34	"	"	"	1 "	39				
13-37		"	"	"	1 "	31				
13-38		"	"	"	3 papillomas	28				
13-39		"	"	"	Negative					
13-40	"	"	"	"	"					
14-10	"	"	"	"	4 papillomas	28	1+, 3+	0, 0	0, 0	0, 1+
14-11	"	"	"	"	3 "	28	2+, 4+	0, 0	0, 1+	0, 1+
14-12	"	"	"	"	Negative		4+, 4+	0, 0	0, 0	0, 2+

13-71	Nil, control	"	Confluent papillomatosis	12			
13-72	"	"	"	11			
13-73	"	1:10 1:100	" "	15 17			
13-81	"	1:100	Semiconfluent papillomatosis 11 papillomas	18			
		1:1000 1:10,000 1:100,000	Negative "	22			
13-82	"	1:100	Semiconfluent papillomatosis 13 papillomas	18			
		1:1000 1:10,000 1:100,000	Negative "	22			
14-20	"	Undiluted	Confluent papillomatosis "	10			
		1:10 1:100	Semiconfluent papillomatosis 8 papillomas	11 19			
		1:1000	Semiconfluent papillomatosis 14 papillomas	28			
14-21	"	1:100	Semiconfluent papillomatosis 14 papillomas	19			
		1:1000 1:10,000 1:100,000	Negative "	28			

2+ = many discrete papillomas.

1+ = a few discrete papillomas.

4+ = confluent papillomatosis.

* 0 = no papillomas.

3+ = semiconfluent papillomatosis.

First reading in each column made on 12th day and second reading made on 26th day after inoculation.

non-infectious suspensions was at least as good as that of the rabbits receiving infectious suspensions.

A group of 10 domestic rabbits, not included in Table II, were inoculated by scarification with suspensions of non-infectious domestic rabbit papillomas. After a period of observation adequate to be certain that no growths would appear (35 days) these animals were reinoculated by scarification with a suspension of virus-rich cottontail papillomas. All were still fully susceptible and developed diffuse papillomatosis without prolongation of the incubation period. This result emphasized the importance of the route of administration in achieving immunity by means of non-infectious domestic rabbit papilloma suspensions.

In all of the experiments in which active papilloma virus had been used to vaccinate, the possibility was borne in mind that immunity may have resulted from the formation of occult papillomas, even though on repeated and careful examination none could be seen or palpated. In the experiments with non-infectious papilloma suspensions, known to be incapable of causing either infection or immunity when applied to the scarified skin, this possible explanation of the immunity induced by intraperitoneal inoculation could be rather conclusively eliminated.

Immunization of Cottontail Rabbits by the Intraperitoneal Administration of Infectious and Non-Infectious Suspensions of Cottontail Rabbit Papillomas.—Experimentally produced papillomas of cottontail rabbits usually contain virus that is readily demonstrable by the infection of other rabbits. Rarely, however, they prove non-infectious as do the majority of those from domestic rabbits. The papillomas from cottontail rabbit 9-18 were of this type. It seemed of interest, in view of the findings with non-infectious domestic rabbit papillomas, to determine whether non-infectious cottontail papillomas would also immunize.

Each of 5 cottontail rabbits was injected intraperitoneally twice at 8 day intervals with 2 cc. of the supernatant fluid from a 5 per cent suspension of non-infectious glycerolated papillomas from cottontail rabbit 9-18. For comparison a second group of 5 cottontail rabbits in the experiment received similar injections of a suspension of virus-rich cottontail rabbit papillomas. The skin at the sites of inoculation was treated and excised as described in the experiments with

domestic rabbits. No papillomas resulted. The animals were tested for immunity to papillomatosis 21 days after their second immunizing injection by inoculating their shaved scarified abdominal skin in the usual way with virus. 3 control cottontail rabbits were inoculated at the same time.

Sera of some of the vaccinated rabbits, drawn just before the test for immunity, were studied for their capacity to neutralize papilloma virus as previously described.

TABLE III

Immunization of Cottontail Rabbits to Papillomatosis by Means of Infectious and Non-Infectious Papilloma Suspensions

Cotton- tail rabbit No.	Vaccination (2 intraperitoneal injections 5 per cent suspension glycerolated papillomas)			Test for immunity (massive inoculation of scarified skin with 0.5 per cent suspension infectious papillomas)		Neutralization of papilloma virus by sera of vaccinated cottontail rabbits		
	Rabbit source	Infectious for skin	Papillomas resulting	Result	Incubation period days	Serum dilution		
						1:2	1:5	1:20
13-55	Cottontail 9-18	No	None	2 papillomas	40	0, 1+*	0, 1+	1+, 3+
13-56	" "	" "	" "	1 papilloma	40	0, 1+	0, 1+	0, 3+
13-57	" "	" "	" "	24 papillomas	18	1+, 4+	0, 3+	1+, 3+
13-58	" "	" "	" "	2 "	26			
13-59	" "	" "	" "	24 "	26			
13-60	" 12-34	Yes	" "	6 "	26	0, 0	0, 0	0, 2+
13-61	" "	" "	" "	3 "	26	0, 0	0, 0	1+, 3+
13-62	" "	" "	" "	1 papilloma	26	0, 0	0, 0	0, 0
13-63	" "	" "	" "	4 papillomas	27			
13-64	" "	" "	" "	1 papilloma	27			
13-65	Nil, control			Confluent pap- illomatosis	8			
13-66	" "	" "	" "	" "	8			
13-70	" "	" "	" "	" "	8	4+, 4+		

* Same significance as in Table II.

The results of the experiments with cottontail rabbits are outlined in Table III.

All 10 of the vaccinated cottontail rabbits proved partially resistant to papillomatosis. They developed only few papillomas after prolonged incubation periods. So far as could be judged by the test for active immunity, the suspension of non-infectious cottontail papil-

lomas immunized practically as well as the one which was highly infectious. Slightly better neutralizing antibodies were, however, developed by the animals receiving the virus-rich suspension than by those vaccinated with the non-infectious suspension.

DISCUSSION

Multiple intraperitoneal injections of either infectious or non-infectious glycerolated rabbit papilloma suspensions immunized domestic and cottontail rabbits to papillomatosis. This immunity was achieved without detectable infection of tissues in which the virus causes lesions. Antibodies capable of neutralizing the virus were demonstrable in the sera of vaccinated animals. Any possible immunizing effect of viable papilloma tissues, which might conceivably survive in the peritoneum for a time, was eliminated from consideration by using only suspensions of growths that had been stored in 50 per cent glycerol for 2 months or longer. There is every reason to suppose that the immunity, shown by rabbits vaccinated with infectious papilloma suspensions, developed as a result of the virus they received intraperitoneally. If this supposition is correct, then the immunity produced by non-infectious papilloma suspensions must likewise be considered to have resulted from virus administered. That this virus was effectively masked, perhaps by combination with a neutralizing substance or by alteration to a non-infective phase, was indicated by the failure of the non-infectious suspensions to infect when applied to scarified rabbit skin. The masking, whatever its nature, was not sufficiently complete, however, to alter seriously the antigenic properties of the virus. When given intraperitoneally the non-infectious virus material, like that which was fully infectious, was capable not only of increasing the resistance of rabbits to papillomatosis but of eliciting the formation of specific virus-neutralizing antibodies. It was, in effect, a biologically inactivated virus vaccine.

The outcome of these immunization experiments makes it clear enough that the usual non-transmissibility of papillomatosis serially in domestic rabbits is due to no lack of virus in the domestic rabbit papillomas. It is referable instead, to the efficient masking, in some unknown fashion, of the virus they contain. It seems possible that this masking of virus is the end-result of a host-parasite antagonism

and may represent a defense utilized by the domestic rabbit against unrestrained parasitism by the agent of papillomatosis.

The present experiments, with those of Kidd, Beard, and Rous (4) on the development of papilloma virus-neutralizing antibodies by rabbits bearing transplanted carcinomas derived from papillomas, demonstrate that papilloma virus, though non-detectable by the usual infection test, can and does induce an immunity response. They furthermore demonstrate that the inactive virus does not necessarily have to be associated with actively proliferating epithelial tissues in order to induce this result.

SUMMARY

Two intraperitoneal injections of either infectious or non-infectious rabbit papilloma suspensions actively immunize rabbits against papillomatosis. The capacity of the non-infectious suspensions to immunize is considered as evidence that they contain papilloma virus even though none can be demonstrated by the usual infection test.

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STUDIES ON EXPERIMENTAL HYPERTENSION

II. THE EFFECT OF RESECTION OF SPLANCHNIC NERVES ON EXPERIMENTAL RENAL HYPERTENSION*

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The original purpose of this investigation was to determine whether complete sympathectomy would prevent or cure the type of experimental renal hypertension which can be produced in normal dogs by partial clamping of the renal arteries (1). Bacq, Brouha and Heymans (2) claim that ablation of the sympathetics in the dog can prevent the hypertension that follows denervation of the carotid sinus and section of the depressor nerves. The integrity of the vasomotor system is evidently necessary for this type of hypertension. It was felt therefore that total sympathectomy, before and after the clamping of the renal arteries for the production of hypertension, would also give some estimate of the importance of the vasomotor nervous mechanism in the development of the renal type of hypertension.

Before the work had progressed very far it was reported (3-5) that a lowering of the blood pressure occurs in some human cases of hypertension as a result of excision of the thoracic portion of the splanchnic nerves alone. Back of this work was evidently the idea that the vasomotor nervous mechanism affecting the splanchnic zone alone could be an important determining factor in the pathogenesis of various types of hypertension. It was decided therefore first to test the effect of excision of the splanchnic nerves in the prevention and cure of experimental renal hypertension.

EXPERIMENTS

In four normal dogs the entire thoracic portion of the splanchnic nerves and the lower four dorsal sympathetic ganglia were excised on both sides at one opera-

* This study was supported by the Beaumont-Richman-Kohn Fund.

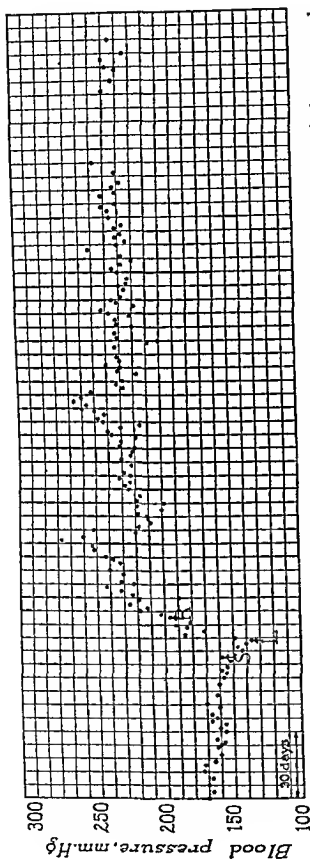
tion. The intrathoracic route was used, with the animal under ether anesthesia and artificial respiration. The incision was made in the 11th interspace (the dog has 13 ribs) and the proper exposure secured by packing and retraction. A small electric lamp at the end of a sterile metal holder was inserted into the chest whenever necessary to aid the dissection and excision of the nerves. After a variable period following this operation the renal arteries were partially clamped in the way previously described (1) for the production of hypertension.

Blood pressure determinations were made frequently as before (1), by the van Leersum carotid loop method, which gives only the systolic pressure. In addition, however, for the purposes of a check by a method that is more objective than the van Leersum method, so called mean blood pressure values were obtained at intervals by direct puncture of the femoral artery. This was done with a 21 gauge needle which was connected to a mercury manometer by means of pressure tubing filled with 4 per cent sodium citrate. In both normal and hypertensive periods these mean pressure readings were always somewhat lower than those obtained by the van Leersum carotid loop method, but the values corresponded fairly closely and significant changes were detected by both methods.

Text-figs. 1 and 2 illustrate the results which were obtained in two of the dogs (2-28 and 2-35), and are illustrative of the group. They show that the usual rise of blood pressure which follows the production of renal ischemia also occurs in animals with splanchnic nerves and lower four dorsal sympathetic ganglia excised. The rise of blood pressure was of the usual order and it has persisted for many months. In these animals there was no accompanying disturbance of renal function, as indicated by tests for urea clearance, quantity of nitrogenous metabolites in the blood and output of phenolsulfonephthalein in the urine. Similar results were obtained in two more animals (2-40 and 2-41).

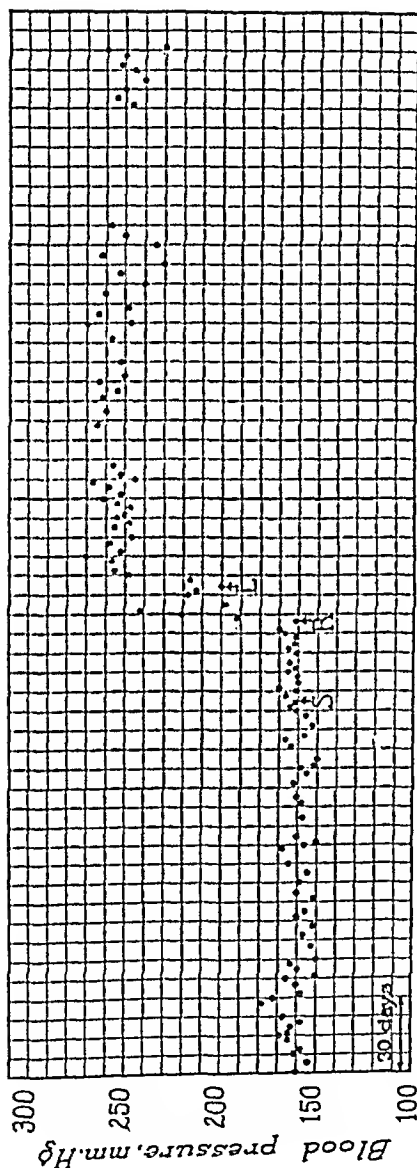
In two other dogs (1-51 and 2-37) the clamping of the renal arteries was purposely made very great but not quite complete. The result was that these animals developed not only the rise of blood pressure but also serious impairment of renal function and clinical uremia. They died a few days after the operation. Text-fig. 3 illustrates the blood pressure and chemical changes in the blood of one of these dogs (1-51).

The results in both groups demonstrate that bilateral excision of splanchnic nerves and lower four dorsal sympathetic ganglia does not prevent the rise of blood pressure and other effects (1) that may follow the production of various degrees of renal ischemia in dogs.



TEXT.-FIG. 1. Dog 2-28. Weight 16 kilos. S, bilateral excision of thoracic portion of splanchnic nerves and lower four dorsal sympathetic ganglia. L, moderate constriction of left main renal artery by means of special sil-ver clamp (1). R, moderate constriction of right main renal artery.

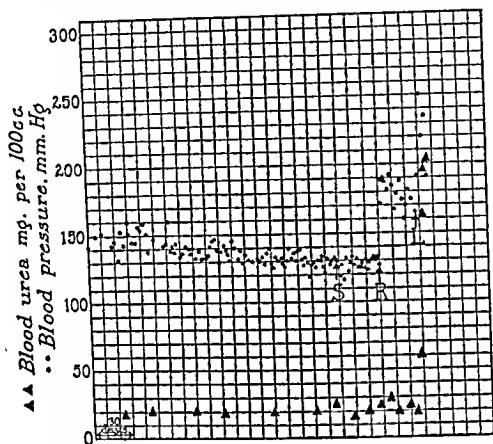
The blood pressure rose in the usual way (1) following the production of renal ischemia and has remained elevated for 9 months. The animal is still alive.



TEXT-FIG. 2. Dog 2-35. Female. Weight 22 kilos. S, bilateral excision of thoracic portion of splanchnic nerves and lower four dorsal sympathetic ganglia. R, moderate constriction of right main renal artery by means of special silver clamp (1). L, moderate constriction of left main renal artery.

The blood pressure rose, following the production of renal ischemia, and has remained elevated for 7 months. The animal is still alive.

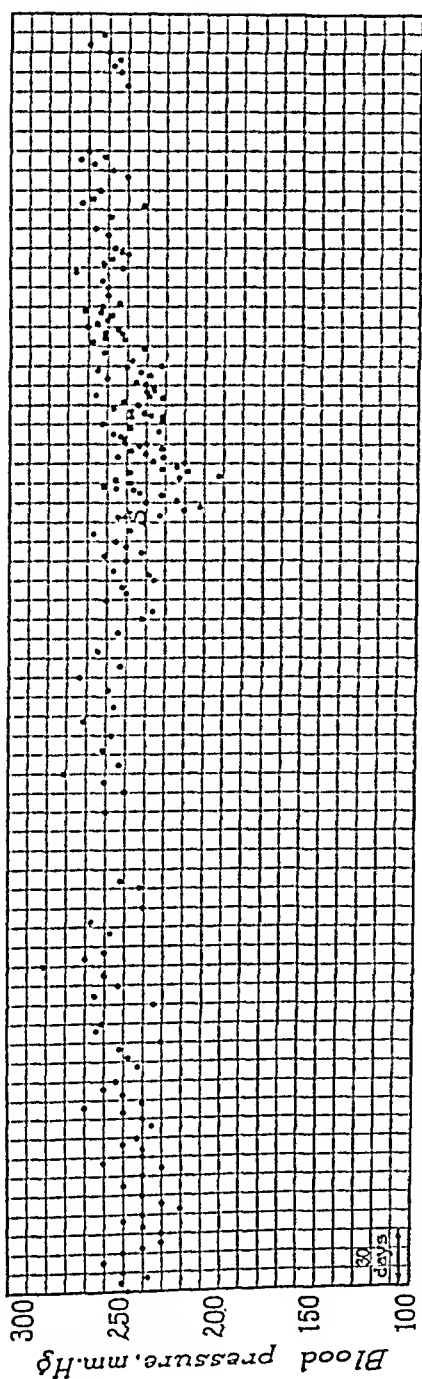
The next step was to determine the effect of excision of splanchnic nerves and lower four dorsal sympathetic ganglia on experimental renal hypertension that had been in existence for a variable period. This is comparable to the curative method used in human beings.



TEXT-FIG. 3. Dog 1-51. Female. Weight 16 kilos. S, bilateral excision of thoracic portion of splanchnic nerves and lower four dorsal sympathetic ganglia. R, severe constriction of right main renal artery by means of a special silver clamp (1). L, severe constriction of left main renal artery.

The blood pressure rose after each clamping. The concentration of urea nitrogen, non-protein nitrogen and creatinine in the blood rose steadily until death. The animal became comatose and developed convulsions 2 days after the clamping of the second renal artery. This state continued until death which occurred 6 days after the constriction of the left renal artery.

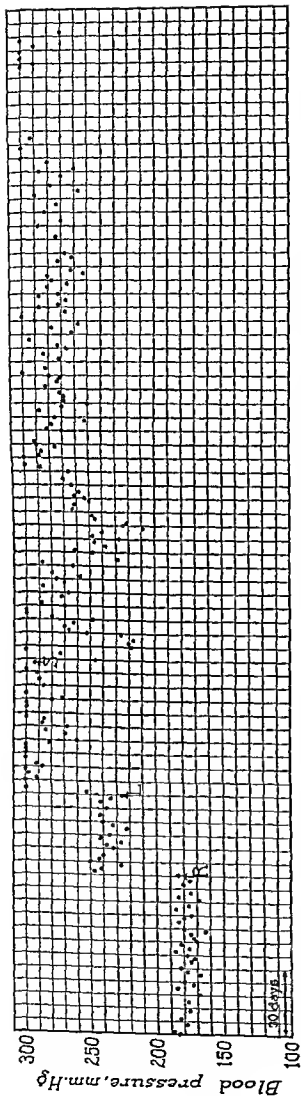
Text-fig. 4 (dog 5-9) shows the effect of excision of the thoracic portion of the splanchnic nerves and lower four dorsal sympathetic ganglia on persistent experimental renal hypertension that had been present for nearly 4 years. During a period of a few weeks following the excision of the nerves, the blood pressure varied considerably and at times was lower than during the period immediately preceding this.



TEXT-FIG. 4. Dog 5-9. Female. Weight 14 kilos. S, bilateral excision of thoracic portion of splanchnic nerves and lower four dorsal sympathetic ganglia.

The systolic blood pressure of this dog was elevated for 4 years before the excision of the splanchnic nerves, due to the production of bilateral renal ischemia. In this text-figure are given the pressures for 13 months before the excision of the splanchnics. The pressures for the 1st year following production of bilateral renal ischemia are given in a previous publication (1). During the 2 year interval the pressure remained elevated to the same degree as in the 2 years that have been recorded in the charts.

For 8 months following the excision of the splanchnics and the lower four dorsal sympathetic ganglia, the pressure has remained elevated to about the same level as during the 4 years prior to this operation. The animal is still alive.



TEXT-Fig. 5. Dog 2-10 Male. Weight 20 kilos. R, moderate constriction of right main renal artery by means of a special silver clamp (1). L, moderate constriction of left main renal artery. S, bilateral excision of intrathoracic portion of splanchnic nerves and lower four dorsal sympathetic ganglia.

For a period of about 3 months following this operation the blood pressure was very irregular and, at times, dropped considerably, but never reached normal levels. For the past 6 months, the blood pressure has been as high as it was before the excision of the splanchnics. The animal is still alive.

operation, but it soon returned to the original level and has now remained elevated for 8 months.

Text-fig. 5 (dog 2-10) shows the effect of excision of the splanchnic nerves and lower four dorsal sympathetic ganglia in an animal in which the elevation of blood pressure had been present for only 3 months. This animal also showed some lowering of the blood pressure and considerable variation of the pressure for a short period following the operation, but the pressure soon returned to the pre-operative hypertensive level and has remained elevated for 9 months. No significant permanent lowering of blood pressure occurred in two other hypertensive dogs (6-0 and 1-17) following excision of the splanchnic nerves and lower four thoracic sympathetic ganglia.

SUMMARY AND DISCUSSION

Excision of the thoracic portion of the splanchnic nerves and the lower four dorsal sympathetic ganglia on both sides failed to prevent the development of persistent hypertension which, in dogs, follows the production of renal ischemia by partial clamping of the renal arteries (1). In dogs with this type of experimental renal hypertension existent for varying lengths of time (up to about 4 years), excision of the splanchnic nerves and the lower four dorsal sympathetic ganglia failed to effect any degree of permanent lowering of the blood pressure.

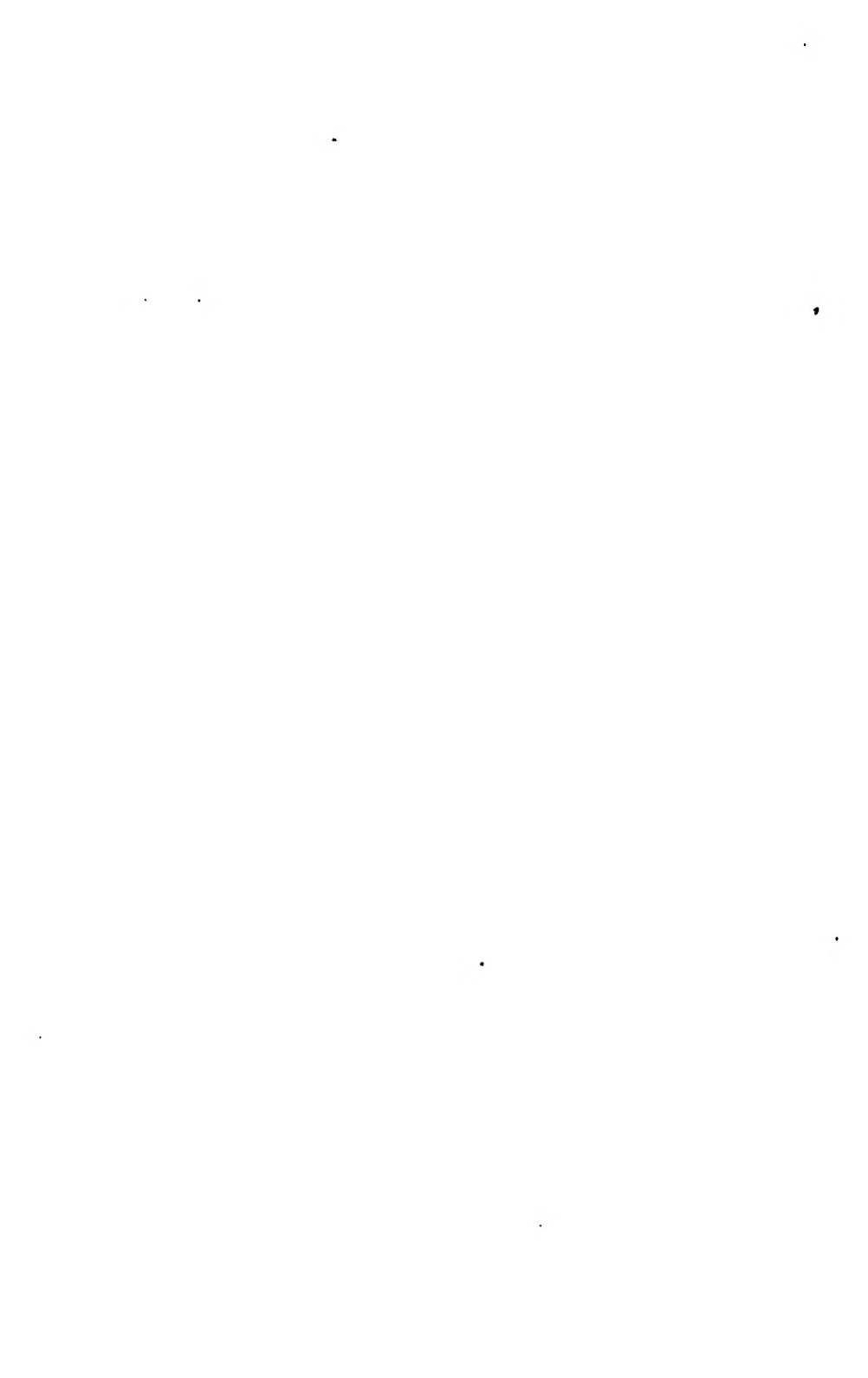
For the dog, at least, these results tend to minimize the importance of the splanchnic vasomotor mechanism in the pathogenesis of renal hypertension. This is in agreement with the conclusions of Prinzmetal and Wilson (6) and of Pickering (7) about the part played by the vasomotor system in human hypertension. It is also in agreement with the work of Page (8), and of Collins (9), who showed that in dogs excision of the extrinsic renal nerves alone does not prevent experimental hypertension due to renal ischemia. Although the results of this investigation fail to give experimental support for the operation that is being practised on human beings with hypertension, yet they do not necessarily controvert the reports of beneficial effects in some cases of human hypertension. Further study of the effects on man is necessary before the results of this operation can be adequately evaluated.

CONCLUSION

In dogs, excision of the thoracic portion of the splanchnic nerves and the lower four dorsal sympathetic ganglia, on both sides, does not prevent, cure or permanently lower in any degree experimental renal hypertension produced by renal ischemia.

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IMMUNOLOGICAL AND CHEMICAL INVESTIGATIONS OF VACCINE VIRUS

VI. ISOLATION OF A HEAT-STABLE, SEROLOGICALLY ACTIVE SUBSTANCE FROM TISSUES INFECTED WITH VACCINE VIRUS

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A specific flocculation occurs when the serum of a rabbit recently recovered from a vaccinal infection is mixed under proper conditions with an emulsion of tissues infected with vaccine virus (1, 2). Craigie (3) has shown that this flocculation is due to the agglutination of suspended particles, notably elementary bodies, and to the precipitation of soluble antigens. The present paper deals with experiments performed in order to obtain information regarding the nature of one of the soluble antigens.

Craigie has demonstrated that there are apparently two soluble antigens which participate in the vaccinal precipitation reaction (4, 5); one of them, designated as L, is destroyed by heat and formaldehyde, while the other, designated as S, is not affected by these agents. The nature of the heat-stable antigen has already been the subject of investigation, and methods have been described by which it can be freed to some extent from other substances present in extracts of tissues infected with vaccine virus. Smith (6) boiled a saline extract of testicular tissue infected with virus at pH 5.5, 8.0, and 7.0 successively, and removed the coagulated protein at each step. He found that by this means the quantity of protein in an extract could be reduced greatly without materially affecting the precipitating titer. The resulting solution gave negative or weak reactions for protein, but a strong Molisch reaction. In view of these results the suggestion was made that the precipitating antigen might be either a carbohydrate or protein, but that it resembled the bacterial baptenes in its action. Ch'en (7) employed the technic of Smith as a preliminary step, and then purified the active substance further by repeated precipitation with alcohol-ether and alcohol, and by dialysis against running tap water. He obtained a white powder which gave weak reactions for protein and a strong Molisch reaction, and which yielded a precipitate when mixed in a dilution of 1:640 with immune serum. He concluded that the precipitinogen was a poly-

saccharide. Craigie (8) has observed that both L and S antigens are precipitated at a pH of 4.6 from aqueous solutions having a low concentration of electrolyte.

In none of the work already described has the purification of the heat-stable antigen or antigens been carried very far, and the exact nature of the active substance or substances still remains in doubt. We have, therefore, attempted to isolate a stable antigen¹ from tissues infected with vaccine virus in order to study its chemical properties and immunological reactions.

Methods and Materials

Virus.—The C. L. strain of vaccine virus was used.

Extracts of Dermal Vaccine Virus.—Rabbits were inoculated dermally with vaccine virus in the form of a suspension of washed elementary bodies. 3 days later the animals were sacrificed, and the virus was harvested into 30–35 cc. of buffer solution (0.004 M, citric acid-sodium phosphate, pH 7.2). From this emulsion the virus and other particulate material were removed by centrifugation and by filtration through a Seitz disc. The clear, slightly yellow filtrate contained the soluble antigens.

Extracts of Testicular Vaccine Virus.—Rabbits were inoculated intratesticularly with an emulsion of bacteria-free virus. 3 days later the testicles were removed aseptically; each pair was ground with alundum and emulsified in 100 cc. of Locke's solution. The emulsion was placed in a sterile flask and ether was added to prevent bacterial growth. After incubation for 5–7 days at 37°C., the ether was removed, and then the emulsion was centrifuged. The supernatant fluid containing the soluble antigens was saved. By this means an extract of virus-infected tissues was obtained free from the products of bacterial infection.

Hyperimmune Flocculating Serum.—The manner in which hyperimmune anti-vaccinal rabbit serum is prepared has already been described (3, 9). Serum against the S antigen was prepared by repeated inoculation of rabbits with a suspension of elementary bodies that had been boiled for 30 minutes; three successive intraperitoneal injections of 1.0, 2.0, and 3.0 cc., respectively, were given at intervals of 1 week; the animals were bled 7 days after the last injection. Rigid precautions were observed in order to prevent the accidental infection of the animals with active vaccine virus (10). Serum prepared in this manner and diluted 1:32 or 1:64 gave precipitates with solutions of S antigen in crude extracts; all of its precipitating activity was removed by absorption with S antigen. L serum was

¹ The word antigen used in connection with our purified material signifies a serologically active substance; as yet we have not had the opportunity of ascertaining whether our material is an antigen in the sense that it excites the production of antibodies when injected into animals.

obtained by absorption with S antigen of LS serum from animals hyperimmunized with active vaccine virus; by this means the S antibodies were removed from the serum which then reacted only with L antigen.

Precipitin Reactions.—Each step in the process of purification of the stable, serologically active substance was controlled and guided by the precipitin reaction previously described (3, 9).

EXPERIMENTAL

Many studies of the chemical and immunological properties of an antigen can be carried out only when the substance has been obtained in a pure state. In this communication a method is described by which a stable substance, probably a protein, has been prepared in a relatively pure form from extracts of tissues infected with vaccine virus, and certain observations regarding the chemical and serological properties of the purified material are recorded.

Preparation of a Stable, Serologically Active Substance from Extracts of Dermal and Testicular Vaccine Virus.—Approximately 500 cc. of an extract of dermal vaccine virus prepared as described above were boiled at pH 7.0 for 5 minutes. A slight precipitate formed which was thrown down by centrifugation and discarded. The supernatant fluid was brought to 50 per cent saturation with ammonium sulfate by the gradual addition of an equal volume of the saturated solution of the salt. A heavy precipitate formed; after standing for an hour this was thrown down by centrifugation and the supernatant fluid was discarded. The precipitate was dissolved in approximately 75 cc. of 0.02 M buffer solution at a pH of 7.2; it was then placed in a cellophane bag and dialyzed against running water for 2 days. By the end of that time a slight precipitate of a water-insoluble material had formed which was removed by centrifugation. The clear supernatant fluid was then brought to 25 per cent saturation with ammonium sulfate by the addition of an appropriate quantity of the saturated solution. A moderately heavy precipitate formed which was separated in the centrifuge and discarded. The supernatant fluid was brought to 50 per cent saturation with ammonium sulfate; the precipitate that formed was collected and dissolved in 30–40 cc. of buffer solution, and the solution again was freed of salts by dialysis. To the solution was added 9 volumes of cold neutral alcohol. A flocculent precipitate formed immediately. After standing overnight the mixture was centrifuged; the supernatant fluid was discarded; the precipitate was dissolved in 50 cc. of water, and the alcohol was removed. To the solution 5 cc. of 0.05 M buffer, pH 4.6, were added. A precipitate formed at once; this was thrown down by centrifugation and after removal of the supernatant fluid was dissolved in a buffer solution at pH 7.2. The supernatant fluid was kept overnight in the cold room; by the following morning a slight precipitate had appeared in it which was collected, dissolved in a buffer solution of pH 7.2, and added to the solution of the original precipitate. About 10 to 25 per

cent of the antigen remained in solution at pH 4.6; this amount was lost. The reaction of the solution was adjusted to pH 7.8 and the solution was boiled. The slight precipitate which formed was removed. The reaction was then adjusted to pH 6.0, and the solution was again boiled. A heavier precipitate formed which was likewise discarded. The water-clear supernatant fluid was dialyzed against distilled water until free from electrolytes. It was then frozen and desiccated *in vacuo* over calcium chloride while in the frozen state. This procedure yielded about 15 mg. of a voluminous white substance.

The manner of preparation of a stable antigen from extracts of testicular vaccine virus was similar to that described above. The serological activity of the product obtained, however, tended to be somewhat lower than that of the active material secured from dermal virus.

Serological Characteristics of the Purified Substance

The purified material in a dilution of 1:640,000 gives rise to a precipitate in the presence of immune serum. This occurs with an S serum, but not with a LS serum from which the S antibodies have been absorbed. Experiments to determine whether the substance is capable of stimulating the formation of antibodies against itself have not yet been carried out. It may be noted, however, that boiled elementary bodies containing no L antigen, give rise to antibodies against our stable substance.

Chemical Characteristics of the Purified Substance

The purified substance obtained by the method described above is white and voluminous, tends to cohere, and consequently resists disintegration by grinding. It dissolves readily in distilled water to make a clear colorless solution which forms a persistent foam when shaken. It is almost completely precipitated from an aqueous solution at a pH of 4.6. It is soluble in 80 per cent alcohol to which a small amount of hydrochloric acid has been added, but is insoluble in neutral 80 per cent alcohol. It is salted out by ammonium sulfate when the concentration of the salt is between 25 and 50 per cent saturation. A solution of the antigen gives an intense Molisch reaction and yields a heavy precipitate with trichloroacetic acid. It is not affected by boiling for brief periods between pH 6.0 and 8.0. The purified antigen contains 16.5 per cent nitrogen.²

² Analysis carried out by Dr. W. F. Goebel.

Effect of Enzymes on the Purified Substance

In order to obtain further information regarding the nature of the active substance, the action of certain enzymes upon it was observed.

Trypsin and Chymotrypsin.—Crystalline trypsin and chymotrypsin³ were dissolved in 0.02 N HCl in the proportions of 5 mg. of enzyme to each cubic centimeter of solution. A solution of commercial (Fairchild) trypsin was prepared by dissolving 50 mg. of dry substance in each cubic centimeter of 0.02 N HCl. In the tests, 0.5 cc. of each of the enzyme solutions was mixed with 0.5 cc. of a solution containing 0.5 mg. of the serologically active substance. The reaction was adjusted to pH 8.4 and the volume made up to 2.0 cc. Ether was added to prevent bacterial growth, and the mixtures were incubated overnight at 37°C. Controls of antigen alone, to determine the effect of incubation at pH 8.4, and of enzyme plus casein, in order to demonstrate the activity of the enzyme, were included in the tests. In all cases the casein was digested, and the precipitating titer of the substance incubated with crystalline trypsin and with chymotrypsin was undiminished, but there was a four- to eightfold reduction in titer of the antigen incubated with commercial trypsin.

In order to show whether the loss in precipitating activity of the substance was due to action of the enzyme on it or to inhibition of the precipitin reaction, the following experiment was performed.

A mixture of the purified substance and enzyme was prepared in the manner described above, and the reaction was adjusted to pH 8.4. It was immediately divided into 2 equal parts, one of which was incubated for 6 hours at 37°C. and then neutralized; the other was neutralized immediately and boiled in order to destroy the activity of the enzyme. The titer of the antigen in the latter mixture was the same as that of the antigen to which no enzyme had been added, while the titer of the antigen in the former mixture which had been incubated at a pH suitable for enzyme activity was reduced eightfold.

Pepsin.—Only commercial enzyme was used to determine the action of pepsin on the purified active substance. A mixture of 0.5 mg. of commercial pepsin and 0.5 mg. of antigen was made, the reaction of the solution was adjusted to pH 2.0, and the volume made to 2.0 cc. The solution was incubated overnight at 37°C. Appropriate controls were included, which indicated that the enzyme was active, and that the antigen was not affected by incubation with buffer. Under the conditions of the experiment there was an eightfold reduction in the activity of the antigen incubated with pepsin.

From the results of the experiment just described it appears that the serological activity of our purified material is greatly reduced by

³ Obtained from Dr. J. H. Northrop.

digestion with commercial trypsin and with commercial pepsin, but that it is resistant to the action of crystalline trypsin and crystalline chymotrypsin.

DISCUSSION

In the present communication a method has been described by which a heat-stable, serologically active substance has been isolated from tissues infected with vaccine virus. If the substance is not absolutely pure, it approaches purity more closely and is decidedly more active—producing a precipitate in a dilution of 1:640,000—than any soluble antigen that has hitherto been isolated from tissues infected with the virus of vaccinia.

The purified active material contains 16.5 per cent nitrogen, and, if the usual factor of 6.25 is used to express the relation of nitrogen to protein, all of the material is accounted for as protein. Indeed, all of the studies so far conducted are consistent with the idea that our substance is an alcohol-soluble protein. It should be noted, however, that it is soluble only in the form of the acid salt and is insoluble in neutral alcohol. It is not precipitated by boiling in a neutral aqueous solution and is soluble in distilled water. That carbohydrate is present is indicated by the fact that the material yields a strong Molisch reaction. It is possible that the carbohydrate may be present as an impurity, yet it is known that certain conjugated proteins contain carbohydrates of constitution and that these give Molisch reactions (11). The fact that digestion of our material with pepsin and commercial trypsin greatly reduces its precipitating activity indicates that the integrity of the protein molecule is essential for its full serological activity. While proteins possessing characteristics similar to those described are not common, they have been isolated from bacteria, *e.g.*, the type specific protein (M) obtained from streptococci by Lancefield (12).

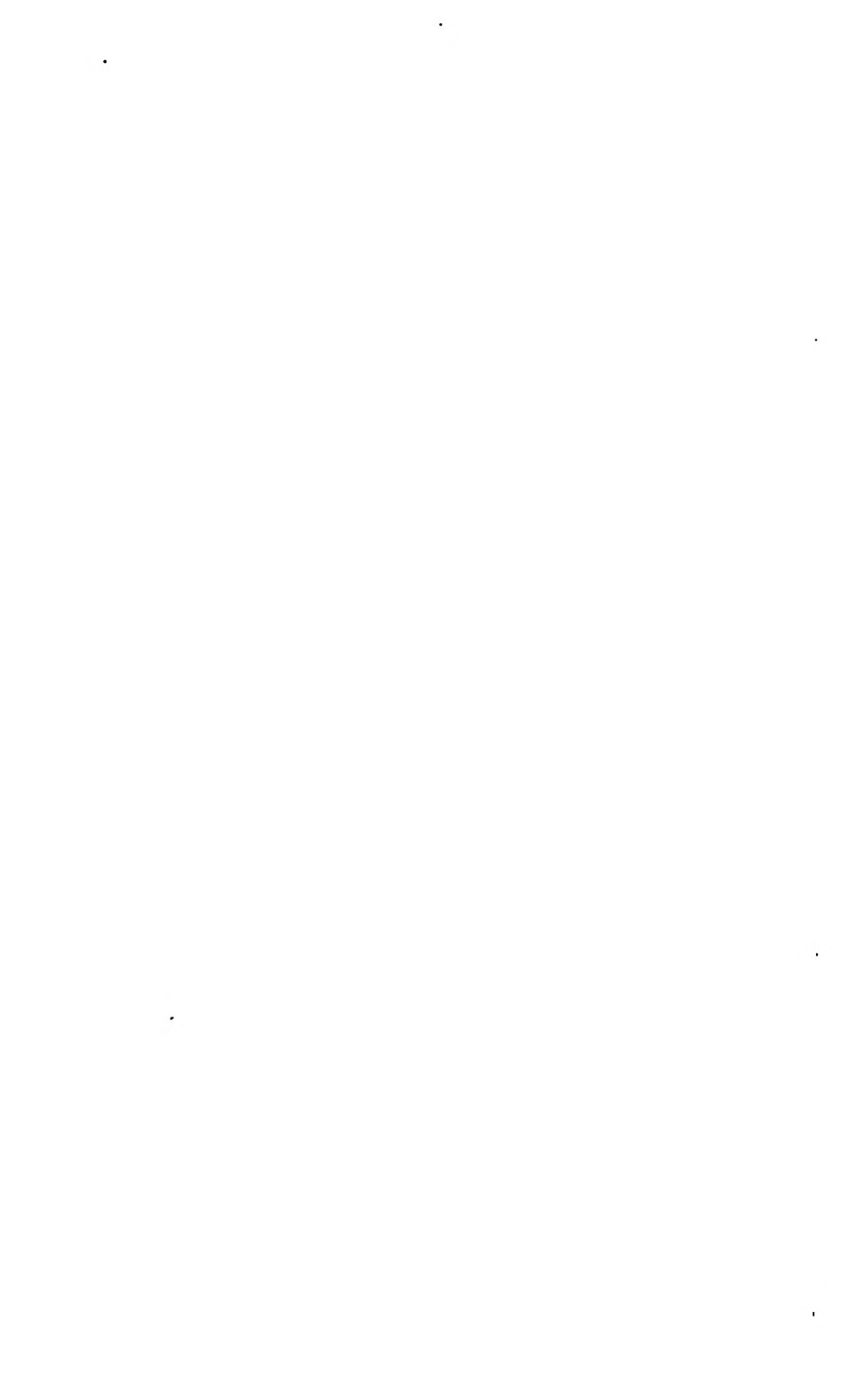
With the present data it is impossible for us to state definitely that our purified material represents only one antigen. Furthermore, the exact relation of our serologically active substance to those described by Smith (6), Ch'en (7), and Craigie (5) is not known, yet all of them possess certain similarities, and, in view of previous work on crude vaccinal extracts by several investigators (2, 5, 9, 13), one is warranted in concluding that they are specifically associated with vaccinia.

SUMMARY

A method has been described by which a stable, serologically active substance has been isolated in a relatively pure state from tissues infected with vaccine virus. It has the characteristics of an alcohol-soluble protein which is not precipitated by boiling in a neutral aqueous solution. In a dilution of 1:640,000 it gives a precipitate when mixed with a serum containing antibodies against Craigie's S antigen of vaccine virus, but no visible reaction occurs when it is mixed with serum depleted of S antibodies by means of absorption.

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THE ANTIBODY RESPONSE OF HUMAN SUBJECTS VACCINATED WITH THE VIRUS OF HUMAN INFLUENZA

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Previous studies (1, 2) have revealed that the virus of human influenza is capable of infecting mice and ferrets only when introduced into the respiratory tract. When mice are inoculated subcutaneously or intraperitoneally infection does not occur, but with repeated inoculations in this manner the animals develop an active resistance to infection by the respiratory route. Similarly, though to a somewhat less extent, ferrets, while exhibiting no evidence of infection from subcutaneous injections of active virus, develop circulating antibodies and increased resistance to the virus introduced by way of the nasal passages.

Because of the significance of these observations as a possible guide toward preventive measures, it was important to determine the effects of the introduction of untreated active virus in human individuals. The use of animal tissues as the source of virus for human study is accompanied by certain undesirable features, such as the possibility of protein sensitization, or the introduction of bacterial or extraneous virus contaminants. In order to eliminate these difficulties, the virus derived from infected mouse lungs was introduced into tissue culture medium. The medium was that described by Li and Rivers (3), which consists of minced chick embryo suspended in Tyrode's solution.

Under these conditions the virus multiplied readily (4, 5) and has been carried through 160 subcultures in 11 months. The culture virus maintains its capacity to infect mice and ferrets by intranasal inoculation and also to induce immunity in these animals when administered by subcutaneous and intraperitoneal routes. It was employed for the work described in the present paper. This embodies the results of

titrations of the capacity of the serum of 22 human individuals, before and at intervals up to 5 months after vaccination, to neutralize approximately 1000 lethal doses of the mouse passage human influenza virus as measured by mouse protection tests.

EXPERIMENTAL

The results of studies of Andrewes, Laidlaw, and Smith (6) in England and those of Francis and Magill (7) in the United States have shown by mouse protection tests that the serum of a high percentage of individuals of all ages possesses the capacity to neutralize the human influenza virus. In order to test the effect of vaccination, it was necessary to select so far as possible subjects whose serum beforehand possessed the least neutralizing capacity.

From 60 available volunteers, 23 were chosen for the test. All but 5 were medical students in the third decade of life. The others ranged from 35 to 64 years of age. The supernatant fluid of cultures made as above described from which the cells had been removed by centrifugation at low speed, was used for vaccination. To 11 of the subjects doses of 0.5 cc., 1.0 cc., and 1.0 cc., respectively, were given subcutaneously at weekly intervals, and after a further interval of 2 to 3 weeks an additional dose of 2.0 cc. of the virus-containing fluid was given by the same route. To 5 individuals doses of 1.0 cc., 1.0 cc., and 2.0 cc., respectively, were given subcutaneously at weekly intervals. The remaining 7 subjects were given three successive doses of 0.5 cc. of culture virus intradermally at weekly intervals.

A sample of serum was obtained from each subject before vaccination, before each subsequent injection, and 10 days after the final injection (8). Where possible, serum was again obtained from each volunteer 2 and 5 months after the final vaccinating dose of virus.

The virus used in the protection tests was the mouse passage Puerto Rico 8 strain. The virus was obtained from the lungs of infected mice, which were ground and suspended in 10 per cent normal horse serum in physiological salt solution. After centrifugation at 2000 revolutions per minute for 15 minutes, the supernatant fluid was diluted to a 2 per cent virus concentration. Each serum was tested undiluted and in dilutions of 1:5, 1:10, 1:20, 1:40, 1:80, and 1:160. Serum dilutions were made with physiological saline. To each serum dilution was added an equal quantity of 2 per cent virus suspension; the mixture was incubated at 37°C. for 30 minutes; and 3 mice were then inoculated intranasally with 0.03 cc. of the mixture. The 4 specimens of serum taken at various times from the same individual were subjected to test at the same time, and usually those from 4 individuals were tested together. As a control, the serum of one individual taken 5 months after recovery from influenza was titrated in each experiment.

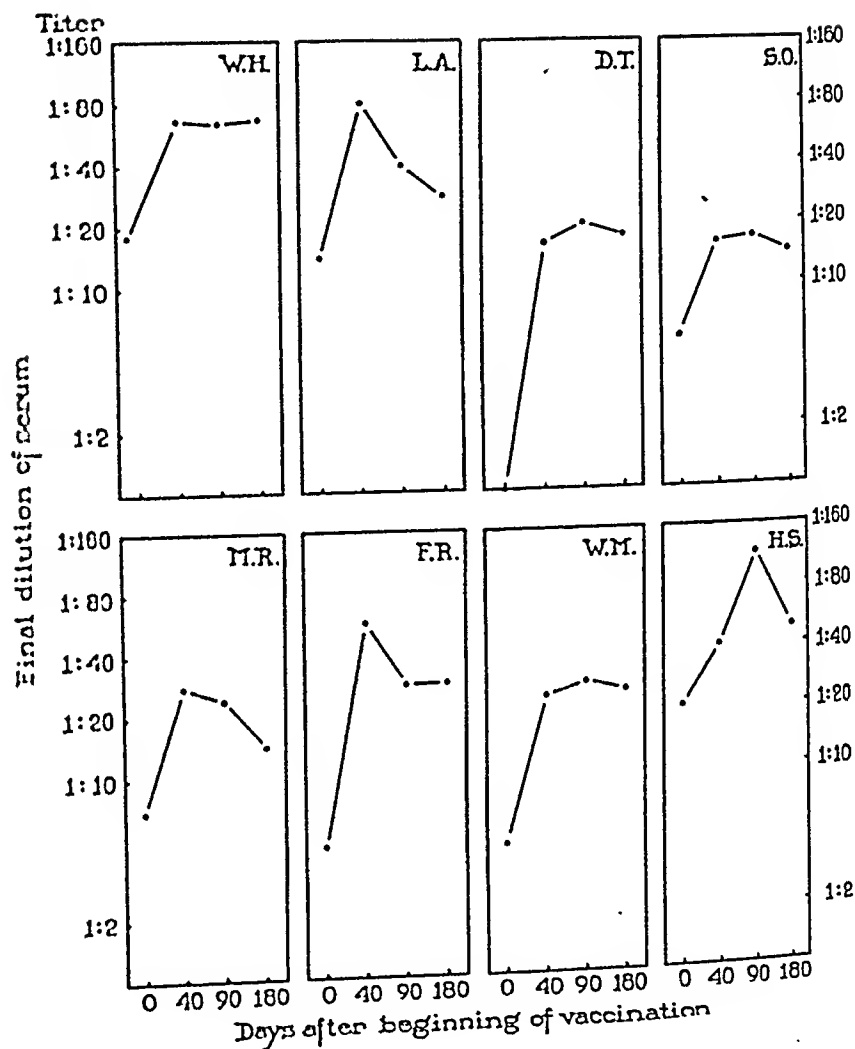
Each was terminated on the 7th day after infection: all surviving mice were autopsied then and the extent of the pulmonary involvement was recorded. The titer of the serum was estimated as the greatest dilution which resulted in a 50 per cent survival of the mice throughout this period. Mice which presented extensive lung lesions at autopsy on the 7th day were considered, however, not to have been protected.

RESULTS

The results were uniform in that the serum of each individual developed an increased capacity to neutralize human influenza virus as a result of vaccination. This increase is not a slow, gradual rise throughout the period of vaccination. On the contrary, the most significant rise in antibodies occurs rather abruptly in the 2nd week. The serum titrations reveal the facts that the antibody levels are highest immediately following the course of vaccination, that in general the same approximate concentration is maintained for 2 months, but that at 5 months a decline in titer is observed (Text-figs. 1, 2, 3). In spite of the fact that a decline in titer occurs, the residual titer of neutralizing antibodies in all but one instance remained at a level well above that of the original.

While the same general trend prevailed in the entire series, considerable variation occurred in the antibody response of different individuals. The height of the titer attained and the rate of decline in titer with the passage of time seem to be functions of the individual subject. Nevertheless, the results suggest that those persons whose serum possessed the most antibody prior to vaccination responded to vaccination with the formation of less additional antibody than subjects whose original titer was quite low. For comparison the sera of three patients who actually suffered from influenza (2) and their sera 3 weeks after recovery and again 5 months after recovery were titrated (Text-fig. 4). In these three cases a sharp antibody rise is evident after 3 weeks' convalescence. The height of antibody in these cases is not strikingly dissimilar to that of the vaccinated group (Text-fig. 5). Moreover, the same tendency to decline after 5 months is observed, though perhaps to a less extent.

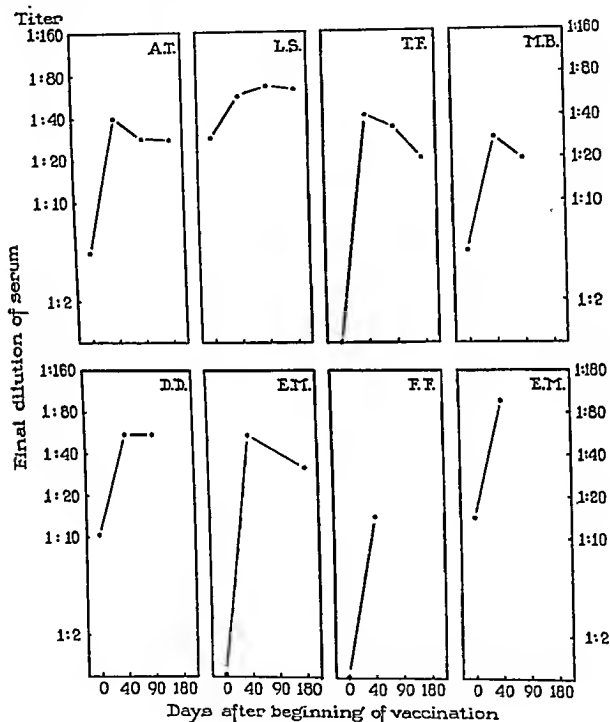
The results in the group of individuals receiving vaccination by the intradermal route appear to follow the same general course as in those inoculated subcutaneously. The mean titer of the serum of the former



TEXT-FIG. 1

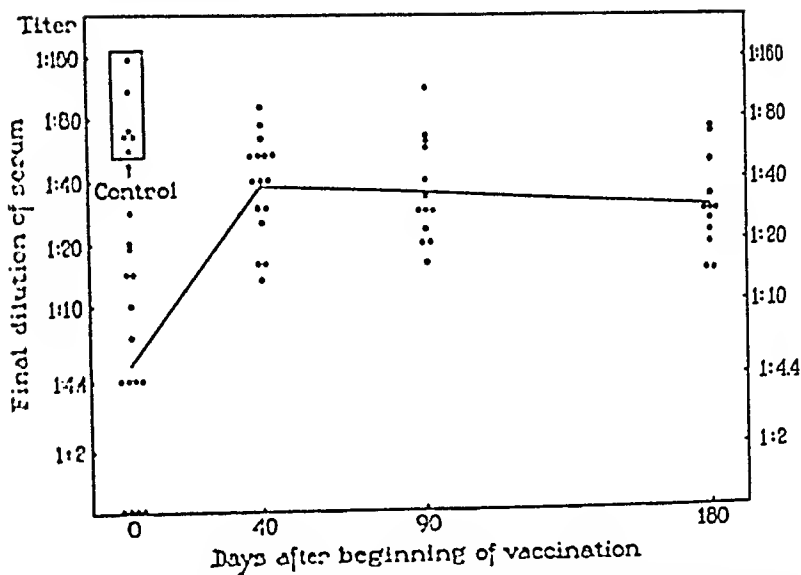
TEXT-FIGS. 1 and 2. Antibody response to subcutaneous vaccination. Each block represents the findings in a single individual vaccinated by the subcutaneous route. The time interval between the first vaccinating dose of virus and the date on which the serum was obtained, representing the end of the period of vaccination, was 40 days. The time intervals are thus measured from the date on which vaccination was begun. The titer of the serum is recorded in terms of final effective dilution.

group before vaccination was approximately 1:10. It increased to about 1:40, a fourfold increase. The mean titer of the subcutaneously vaccinated group was about 1:4 before and 1:40 after, a tenfold



TEXT-FIG. 2

increase (Text-fig. 6). Two modifying factors may play some rôle in this result: first, the total amount of virus administered is smaller; second, these subjects had comparatively high original antibody titers.



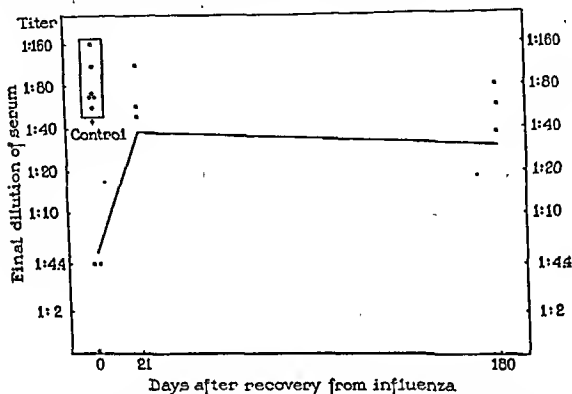
TEXT-FIG. 3. Titers after subcutaneous vaccinations. (Line connects means of groups.) The solid line represents the geometrical mean of all tests with the sera of the subjects vaccinated by the subcutaneous route. The black circles represent the results of individual tests. The titers of the control serum, as measured in different tests, are enclosed in the left upper corner.



TEXT-FIG. 4. Antibody response to natural infection. The titer of the serum of 3 human individuals tested during the acute phase of influenza, 21 days and 180 days later, respectively.

Absence of Unfavorable Reactions Following Vaccination

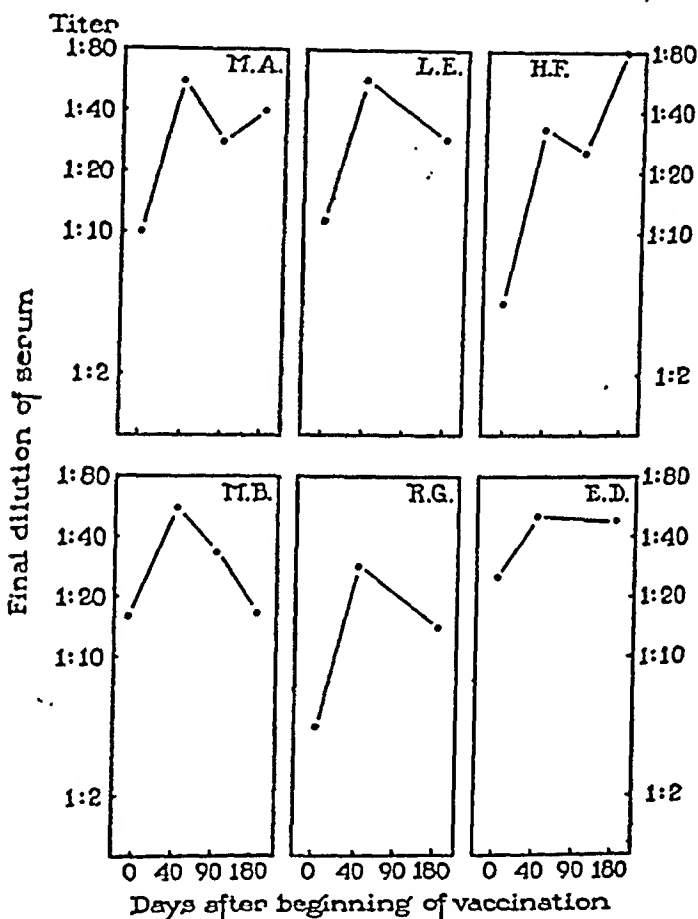
In addition to the knowledge obtained regarding the development and persistence of antibodies following vaccination, it was important to determine whether any ill effects were contributed by the introduction of untreated, active virus. To this end each subject was admitted to the hospital isolation ward for 48 hours at the time of the first injection, and again at the time of the largest (2 cc.) dose. Carc-



TEXT-FIG. 5. Titers after natural infections. (Line connects means of *subcutaneous* groups.) The titers of the serum of 3 patients during the disease and up to 180 days after recovery are shown as black circles. For comparison, a solid line is superimposed, representing the mean titers of the sera taken within a similar time period after the subcutaneous vaccination (see Text-fig. 3).

ful isolation precautions were observed, repeated temperatures were taken, all symptoms were recorded, and examinations of the site of inoculation were made. In those who received the virus subcutaneously no significant elevation of temperature occurred, and the local reactions were inconstant and extremely mild—frequently unnoticed by the subject himself. In those who received virus intradermally more immediate erythematous reaction was observed at the site of injection, but no unpleasant features occurred. Furthermore, two

subjects were inoculated with influenza virus while suffering from common colds. No aggravation of symptoms was noted. One subject was given but one dose of 3 cc. of tissue culture virus subcutaneously. After an asymptomatic interval of 48 hours, nasal and



TEXT-FIG. 6. Antibody response to intradermal vaccination. The titer of serum of individuals, taken at intervals up to 180 days after beginning of vaccination by the intradermal route.

pharyngeal washings were obtained to ascertain whether virus could be recovered from the respiratory tract. The concentrated mucus was inoculated into the nose of a normal ferret; which exhibited no evidence of infection, nor were specific antibodies subsequently

demonstrable in the ferret's serum. This indicates, of course, that virus introduced subcutaneously does not readily find its way to the respiratory tract. Furthermore, Chenoweth *et al.* (9) have vaccinated a large group of human subjects with influenza virus obtained from mouse lung. In no instance was evidence of infection observed.

SUMMARY

Human influenza virus cultivated in tissue culture medium may be administered subcutaneously or intradermally to human individuals without causing evidence of infection. Subjects so treated develop a good titer of circulating antibodies effective against mouse passage virus and, if antibodies were previously present, vaccination stimulates the production of more antibody. The antibodies so induced persist for at least 5 months, although in this period of time some decline in titer may have begun. The antibody response to vaccination parallels both in extent and persistence that occurring as a result of the naturally acquired disease.

The available data do not enable one to evaluate the effect of vaccination in preventing human infection with influenza. It seems not unlikely that the increase in circulating antibody will be accompanied by an increased ability to combat the natural infection.

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INHERITANCE OF RESISTANCE OF MICE TO ENTERIC BACTERIAL AND NEUROTROPIC VIRUS INFECTIONS

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Previous studies from this laboratory have shown that certain factors regulating the resistance of mice to naturally induced *Bacillus enteritidis* infection are inborn (1). The new data to be presented show that, under the experimental conditions, resistance to *B. enteritidis* and resistance to St. Louis encephalitis virus are inherited in a similar and relatively definite manner but independently.

Materials and Technique

The general technique employed in this work has been described (1). It was pointed out that following early experiments on small numbers of mice from the Rockefeller Institute breeding stock, a special breeding colony was started in 1929 with 600 of these animals which were tested and proved free of infection. The diet was changed from a bread and milk ration, producing mortalities following injection of mouse typhoid bacilli generally high (70 per cent), but fluctuating with season, to one producing low and more stable mortalities (37 ± 1.6 per cent). Finally, *B. enteritidis* rather than *B. aertrycke* was used as the test agent. These different experimental conditions, reflected in the different levels of mortality of the two control groups, permit early and later data to be compared qualitatively (1) but not quantitatively (Hill, 2).

The breeding stock was established and has remained free of the infections under investigation and others as determined by continued testing. At the outset it would have been preferable in selecting for possible resistant strains to proceed as with the susceptibles and choose for breeding, unexposed siblings of tested litters rather than the survivors themselves. This error was committed on but a single occasion, at the commencement of the experiment, however, and the rigid measures employed for detecting infected animals were known to be effective. Only the uninfected survivors were mated and the resulting litters tested, found to be uninfected, and then placed in the breeding room. Subsequently, no survivors have been used for breeding.

The mouse colony is subjected to continued search for infectious agents. The occasional sickly mouse is sacrificed and tested for the presence of pathogenic

bacteria or virus. Feces from breeders are tested six to twelve times per year for the presence of mouse typhoid. Susceptibles and resistants are housed together after weaning time. No infection has been found; hence Hill's intimation that typhoid may persist in our colony is without basis (2).

Further Development of Susceptible and Resistant Lines

In the previous paper (1) results were given on six selections and generations of susceptibles and four selections and three generations of resistants from 1929 to June, 1932. Unselected controls showed a relatively stable mortality rate of 37 per cent. Of susceptible lines, all save 1 and 2 were discarded. These had shown a relatively stable rate of approximately 85 per cent from the outset. Other lines subsequently discarded had shown increasing mortality rates with selection. Of resistant lines, only 1 and 2 were retained. These showed rates fluctuating about a 15 to 20 per cent average.

The results of further selective breeding of susceptible lines 1 and 2 and resistant lines 1 and 2 will now be described. Selections for breeding were made as previously, from litters unexposed to infection, thereby insuring against introduction or persistence of the infection in the stock. While other workers (3-8) have bred from survivors of a test infection, our method has been to remove the first and often the second litter from the breeding room at 4 to 6 weeks of age and test each with *B. enteritidis* or virus. If the mortality was maximum or minimum as required, an additional sibling litter was selected and mated brother to sister without testing. First and second litters of the succeeding generation were then tested in the same manner and a third litter chosen for mating. Tests were run as frequently as batches of mice of different generations and different lines became available. The crude results of these tests will first be described to show the chronological progress of the work.

Previous efforts (1) to increase susceptibility and resistance by a more rigid procedure of giving resistants a 10 to 1,000 times greater dose of *B. enteritidis* than susceptibles, commenced July, 1931, were discontinued Oct. 4, 1932, without noticeable success. This result was not surprising in view of earlier tests on mice injected *per os* which had shown that changes in dosage as great as 1,000-fold did not materially influence mortality rates (9).

Subsequent tests, nine in number, from Oct. 4, 1932, to June 2, 1933, in which the standard dose, 5×10^6 of *B. enteritidis*, was given *per os* to both susceptibles

and resistants, showed an abrupt increase in mortality rates of all lines. Susceptibles previously stable at 85 per cent rose to 95 to 100 per cent, resistants from 20 per cent to 50 or 60 per cent, and unselected mice from 37 per cent to 60 or 70 per cent. This disturbing variation was due in all probability to some unknown alteration of environmental factors.

Commencing Dec. 7, 1932, and continuing through Dec. 11, 1933, the bacteria-susceptible and bacteria-resistant lines were tested for their resistance to nasally instilled louping ill virus (10). The first six of these tests have already been reported (1). Of a total of twenty-one tests with different and similar doses within and between runs, twenty showed mortality rates of susceptibles less than those of resistants. Of the total 803 susceptibles tested, 49.4 per cent died as contrasted with 91.7 per cent of 775 resistants.

It will be recalled that selections of mice for breeding had been made entirely on the basis of maximum susceptibility or resistance of progeny to *B. enteritidis*. The susceptible and resistant lines which developed were later found to be susceptible and resistant respectively to *Pasteurella ovicida*, *B. friedländeri*, and pneumococci administered intranasally (1). Accordingly we came to designate the former as bacteria-susceptible (BS) lines and the latter as bacteria-resistant (BR). But now, in 1932 and 1933, when it developed that the bacteria-susceptible lines were resistant to louping ill virus and the bacteria-resistant lines susceptible, it became necessary to complicate the terminology further and to identify the one as bacteria-susceptible-virus-resistant (BSVR) lines, and the other as bacteria-resistant-virus-susceptible (BRVS) lines.

Finding the mortalities of BSVR lines to louping ill virus averaging about 50 per cent and often concentrated entirely in certain litters led us to attempt to develop sub-lines highly resistant to virus (BSVR) and highly susceptible (BSVS) respectively. Parents whose progeny had shown least and greatest mortality following nasal instillation of louping ill virus were selected for further breeding; all others in these bacteria-susceptible lines were discarded. The resulting second litters were tested, further discarding of parents practiced, and a third mating made of parents whose litters showed greatest and least mortality to virus. The resulting litters were weaned, mated brother to sister, and their progeny tested with virus.

At this point it should be restated that no animals given test bacteria or virus were ever bred or placed in the breeding room. Matings were made invariably—with the single exception in 1930 previously recorded—from sibling litters of those tested, never exposed to any infection under investigation.

Litters from parents of the sixth generation of the bacteria-susceptible (BS) lines selected on the basis of susceptibility of progeny to louping ill virus were first tested Dec. 11, 1933, with 85.5 per cent of 69 so called virus-susceptibles (BSVS) dying, as contrasted with 25.8 per cent of 97 virus-resistants (BSVR). Subsequent tests were made with encephalitis virus, St. Louis type.

Encephalitis virus, St. Louis type, was obtained, prepared, and instilled intranasally into the test mice in the same manner as louping ill virus. Its distribution in the animal following this procedure, its clinical and pathological effects have

TABLE I

Comparative Mortalities of Unselected and Selected Lines of Rockefeller Institute Mus musculus albinus Mice Following Test Injections of B. enteritidis, Louping Ill, and Encephalitis, St. Louis Type, Virus

Date	Unselected			Bacteria-susceptible lines (BS)						Bacteria-resistant lines (BR)					
	Tests with <i>B. enteritidis</i>			Tests with <i>B. enteritidis</i>			Tests with virus			Tests with <i>B. enteritidis</i>			Tests with virus		
	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead
1932															
Sept. 7				134 ¹	107	79.9				82 ¹	9	11			
" "				45 ¹	38	84.4				43 ¹	7	16.3			
" "				50 ¹	38	76				37 ¹	8	21.6			
Oct. 4				30 ²	29	96.7				71 ²	17	23.9			
Nov. 1				86	86	100				91	20	22			
Dec. 1				45	45	100				45	26	57.8			
" 7A							A ₅ ³	3	60				5 ³	5	100
" 19							20 ⁴	8	40				20	12	60
" 27							15 ⁴	4	26.7				20	18	90
" "							15	3	20				20	16	80
" 29							15 ⁵	5	33.3				20	11	55
" "							15	6	40				20	8	40
1933															
Jan. 4				41	39	95.1				102	37	36.3			
Feb. 1	28	17	60.7	24	24	100				92	49	53.3			
" 25							40 ⁵	18	45				40	31	77.5
Mar. 2	30	22	73.3	146	140	95.9				131	70	53.4			
" 6							39 ⁶	9	23.1				38	36	94.7
" 16							24 ⁷	8	33.3				22	21	95.5
" "							31 ⁸	4	12.9				30	17	56.7
" 28							44 ⁹	37	84.1				45	45	100
" "							4 ⁹	2	50				4	4	100
Apr. 4	24	16	66.7				72 ⁴	37	51.4	76	46	60.5			
" 20							70 ⁵	34	48.6				78	78	100
" 27										52	19	36.5	175	173	98.9
May 2	22	14	63.6												
" 8							58 ¹⁰	34	58.6				55	55	100
" 18							39 ¹¹	22	56.4				78	78	100
" 29							32 ¹²	23	71.9				60	58	96.7
June 2	19	10	52.6							76	33	43.4			
" 8							49 ⁹	35	71.4				45	45	100
July 6							67 ⁹	44	65.7						
" 27							47 ⁴	22	46.8						
Aug. 16							102 ⁴	39	38.2						
Oct. 13				15	15	93.3				20	11	55			

TABLE I—*Concluded*

TABLE 1

Date	Bacteria-susceptible lines (BS)						
	Sub-line BSVS Bacteria-susceptible- virus-susceptible			Sub-line BSVR Bacteria-susceptible- virus-resistant			
	Tests with virus						
1933							
Dec. 11	69 ⁴ .A	59	85.5	97	25	25.8	
1934							
Feb. 2	67 ⁷ .B	60	89.6	45	10	22.2	
Mar. 12	82 ¹³	59	72	5	0	0.0	
" 20	90	44	48.9	7	1	14.3	
May 1	90	51	56.7	67	9	13.4	
" 16	90	49	54.4	90	5	5.6	
June 14	81	62	76.5	66	38	57.6	
" 27	88	47	53.4	72	39	54.2	
July 12	190	169	88.9	124	57	46	
" 20	83	34	41	91	26	28.6	
Sept. 21	83	72	86.7	149	39	26.2	
" "	139	105	75.5	118	33	28	
Oct. 3	118	95	80.5	99	68	68.7	
" 24	156	129	82.7	124	54	43.5	
" "	106	102	96.2	60	10	16.7	

Explanation of Symbols

¹ Dose for susceptibles 10^6 , for resistants 5×10^7 .

2 " " " and resistants in remaining tests 5×10^6 .

3 " " " " " 1/20.

4 " " " " " 1/10.

5 " " " " " 1/15.

6 " " " " " 1/2.

7 " " " " " 1/50.

8 " " " " " 1/500.

9 " " " " " 1/30.

10 " " " 1/20, for resistants 1/50.

11 " " " 1/10, " " 1/80.

12 " " " 1/10, " " 1/120.

13 " " " and resistants in remaining tests 1/100.

A = louping ill virus employed for virus tests Dec. 7, 1932, to Feb. 2, 1934.

B = encephalitis virus employed for remainder of virus tests.

been described fully elsewhere (11). In the first test 0.03 cc. of a 1 to 50 dilution of mouse brain virus was used (Table I); subsequently the dilution was 1 to 100 and to the best of our knowledge, all variables save the continued process of selection for maximum susceptibility and resistance to this virus were kept uniform. In fifteen tests comprising 1,532 BSVS and 1,214 BSVR mice, 74.2 per cent of the former and 34.1 per cent of the latter died. Variations between tests, however, were considerable.

TABLE II
Effect of Selection on Mortality Following Test Injections

Bacteria-susceptible (BS) line 2										Bacteria-susceptible-virus-susceptible lines (BSVS)										Bacteria-susceptible-virus-resistant lines (BSVR)									
Generation	Progeny tested with <i>B. enteritidis</i>					No. sires	Progeny tested with <i>B. enteritidis</i>				No. dams	Progeny tested with virus				No. sires	No. dams	Progeny tested with <i>B. enteritidis</i>				No. sires	No. dams	Progeny tested with virus					
	No. sires	No. dams	No. litters	No. progeny	No. dead		Per cent dead	No. litters	No. progeny	No. dead		Per cent dead	No. litters	No. progeny	No. dead			Per cent dead	No. litters	No. progeny	No. dead			Per cent dead					
1	1	4	11	59	43	72.8																							
2	2	7	22	97	81	83.5																							
3	4	18	31	167	146	87.4																							
4	8	35	25	130	118	90.8																							
5	16	41	43	212	158	74.5																							
6	12	25	26	136	131	96.3																							
7	2	3	3	12	12	100																							
8	3	3	3	16	15	93.8																							
9																													
10																													
11																													
12																													
Totals.....		829				704	84.9																						

L = louping ill virus employed. No designation = encephalitis virus.

The data on early selections in this and other lines to be described (Tables III, IV, and V) are taken from tables previously published (1).

TABLE III

Effect of Selection on Mortality Following Test Injections

Generation	Bacteria-susceptible (BS) line 1										Bacteria-susceptible (BS) line 1 × 2													
	Progeny tested with <i>B. enteritidis</i>					Progeny tested with virus					Progeny tested with <i>B. enteritidis</i>					Progeny tested with virus								
	No. sires	No. dams	No. litters	No. progeny	No. dead	Per cent dead	No. sires	No. dams	No. litters	No. progeny	No. dead	Per cent dead	No. sires	No. dams	No. litters	No. progeny	No. dead	Per cent dead	No. sires	No. dams	No. litters	No. progeny	No. dead	Per cent dead
1	1	7	24	136	119	87.5																		
2	3	10	18	72	60	83.3																		
3	11	34	63	295	258	87.5																		
4	16	76	52	270	207	76.7																		
5	19	48	54	270	215	79.6																		
6	11	19	19	119	104	87.4																		
7	2	6	6	36	36	100	3	5	7	21 ^L	20	95.2												
8	2	5	5	29	26	89.7	3	6	8	33 ^L	31	93.9												
9							1	2	4	8	8	100												
10							4	14	21	58 ^L	54	93.1	2	2	2	7	6	85.7						
11							1	1	1	3	3	100												
12							3	4	13 ^L	13	100		1	2	2	5	5	100						
Totals.....	1,227	1,025	83	5			262	218	83.2				173	149	86.1							163	19	11.7

L = louping ill virus employed. No designation = encephalitis virus.

TABLE IV

Effect of Selection on Mortality Following Test Injections

Bacteria-resistant (BR) line 1										Bacteria-resistant (BR) line 2														
Generation	Progeny tested with <i>B. enteritidis</i>						Progeny tested with louping ill virus						Progeny tested with <i>B. enteritidis</i>						Progeny tested with louping ill virus					
	No. sires	No. dams	No. litters	No. progeny	No. dead	Per cent dead	No. sires	No. dams	No. litters	No. progeny	No. dead	Per cent dead	No. sires	No. dams	No. litters	No. progeny	No. dead	Per cent dead	No. sires	No. dams	No. litters	No. progeny	No. dead	Per cent dead
1	1	8	18	75	12	16.0							1	12	7	34	11	32.4						
2	10	42	86	460	42	9.1							4	14	16	80	16	20.0						
3	32	134	142	833	130	15.6							4	13	19	119	12	10.1						
4	23	88	90	511	91	17.8	3	3	3	15	12	80.0	4	15	20	84	13	15.5	2	2	2	9	8	88.9
5	23	59	63	343	133	38.8	15	35	47	222	219	98.6	2	8	8	44	7	15.9	1	6	7	25	24	96.0
6	19	49	51	309	171	55.3	19	46	62	284	279	98.2	3	5	5	27	11	40.7	4	8	9	26	26	100.0
7	2	2	2	6	4	66.7																		
Totals.....				2,537	583	23.0				521	510	97.9				388	70	18.0				60	58	96.7

The foregoing data, further analyzed, show the effect of selective breeding on susceptibility and resistance within each line.

Bacteria-susceptible line 2, which at the first selection and generation showed maximum mortalities to *B. enteritidis* of approximately 85 per cent and which remained so without significant change for five selections and generations, showed suddenly, at the sixth selection and generation (Table II), an abrupt rise to 96.3 per cent. This high level of mortality persisted through the eighth generation and likewise through the twelfth generation to date when selections were being made not on the basis of susceptibility to *B. enteritidis* but to virus. Thus, of 134 BSVS mice tested with *B. enteritidis*, 99.3 per cent died and of 94 BSVR, 95.7 per cent died. Selections for susceptibility to virus in the sub-line, bacteria-susceptible-virus-susceptible (BSVS), were accompanied by mortalities increasing from 76.7 per cent at the first selection in the sixth generation to 97.4 per cent at the seventh selection in the twelfth generation, among a total of 1,464 mice tested. Corresponding selections for resistance to virus in the sub-line, bacteria-susceptible-virus-resistant (BSVR), were accompanied by irregular mortalities without trend, averaging 17.4 per cent of 1,054 tested.

Bacteria-susceptible line 1 had an early history similar to line 2. Owing to subsequent events, however (Table III), it was finally discarded. Its 87.5 per cent susceptibility to *B. enteritidis*, noted at the first selection, varied between 76.7 and 100 per cent for six selections and twelve generations and averaged 83.5 per cent (Table III). Selections for high susceptibility to virus were commenced with the seventh generation mice with progeny mortalities averaging 95.2 per cent in the first test. Three further selections on eighth to eleventh generation mice did not change this high level of mortality. Progeny of eleventh generation mice, however, showed only 61.9 per cent mortality to virus, and progeny of twelfth generation mice, 62.5 per cent. No explanation for this alteration was found and the line was discarded.

Bacteria-susceptible line 1, crossed with line 2, was designated line 1 \times 2. It resembled line 2 generally but because of its small numbers was discarded (Table III). Mortalities of progeny following *B. enteritidis* injection were 72.3 per cent in the third generation, 88.9 per cent in the fourth, 89.9 per cent in the fifth, 85.7 per cent in the ninth, and 100 per cent in the tenth and eleventh generations. Selection of a sub-line resistant to virus resulted in mortalities of 15.4 per cent in the first selection, sixth generation, and 16.7 per cent in the eleventh generation, or an average on a total of 163 mice of 11.7 per cent.

Bacteria-resistant line 1, following the first selection, showed a 16.0 per cent *B. enteritidis* mortality of tested progeny, and subsequent selections for four generations did not alter this general level (Table IV). Following the fifth selection, however, progeny of the fifth generation parents showed a 38.8 per cent mortality, 55.3 per cent in the sixth and 66.7 per cent in the seventh generation respectively. Progeny of the fourth generation tested for susceptibility to louping ill virus showed 80 per cent mortality. One selection was followed by an increase

in mortality of the progeny of the fifth generation to 98.6 per cent and a second selection by a mortality of sixth generation progeny of 98.2 per cent. Brother to sister matings within this line were then discontinued and matings made at random within the line.

Bacteria-resistant line 2 behaved in an entirely similar manner. Brother to sister matings were replaced by line matings as in line 1.

To summarize, mice succumbing to or surviving an enteric bacterial or a neurotropic virus infection bear progeny which tend to succumb or survive respectively. The majority of individuals subjected to this progeny test evidence intermediate degrees of resistance which can be modified by repeated selection from generation to generation. Certain of them, however, provided a sufficiently large population be tested, show an initial maximum or minimum susceptibility which remains relatively stable.

The rise in mortality rates which took place abruptly and simultaneously in all lines probably resulted from imperfect control of environmental factors.

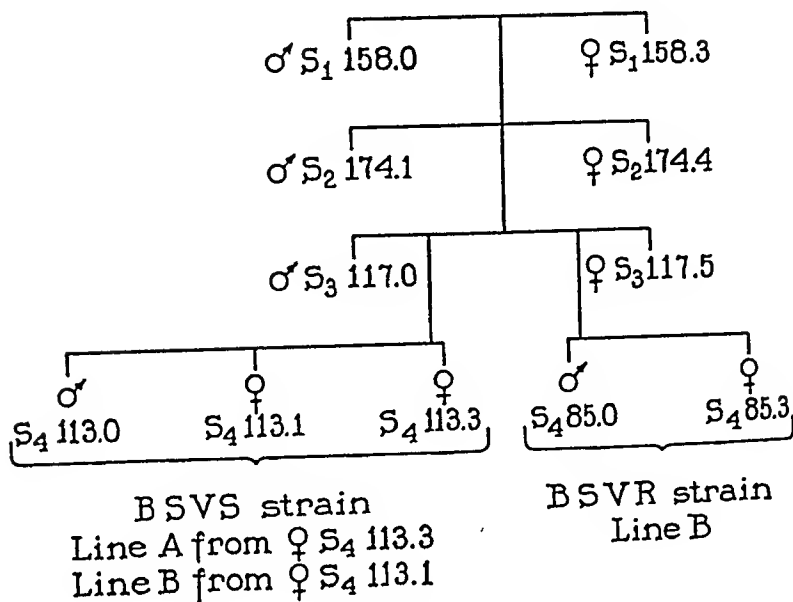
Present Status of Inbred Lines

Rigid selection, testing, and discarding procedures on approximately 13,200 mice for twelve generations from 1930 left us in November, 1934, with three lines, one bacteria-susceptible-virus-susceptible (BSVS), one bacteria-susceptible-virus-resistant (BSVR), and one bacteria-resistant-virus-susceptible (BRVS). The first two came from identical parents and sibling progeny (Text-fig. 1) in bacteria-susceptible line 2 (Table II); the third came from the bacteria-resistant lines 1 and 2. Brother to sister breeding has been practiced throughout in the first two lines; in the BRVS line, it was replaced after the sixth generation by line breeding.

The degree of homogeneity of each line can be appraised to some extent by inspection of the two following sets of data.

The first is the results of susceptibility tests from generation to generation on the immediate forebears of the eventually selected lines and their progeny (Tables V to VII). While Tables II to IV include tests on all progeny within each line, the great majority of which were subsequently discarded, the tables to follow include only tests on siblings in the direct line and their parents.

Susceptible — Line 2



TEXT-FIG. 1

TABLE V

Immediate Family Histories of Inbred BSVS Lines with Respect to Mortalities Following Test Injections of B. enteritidis or Virus

Generation	Line bacteria-susceptible-virus-susceptible A (BSVS)						Line bacteria-susceptible-virus-susceptible B (BSVS)					
	Progeny tested with <i>B. enteritidis</i>			Progeny tested with virus			Progeny tested with <i>B. enteritidis</i>			Progeny tested with virus		
	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead
1	17	14	82.4									
2	44	36	81.8									
3	91	87	95.6									
4	26	24	92.3									
5	27	27	100				18	18	100			
6	11	11	100	12	8	66.7	20	19	95	23	16	69.6
7				24	21	87.5				34	25	73.5
8				23	23	100				37	35	94.6
9	3	3	100	207	183	88.4	8	8	100	144	128	88.9
10	37	37	100	192	168	87.5	11	11	100	155	121	78.1
11	13	13	100	32	27	84.4	16	16	100	24	23	95.8
12	23	23	100	26	25	96.2	12	12	100	13	13	100
Totals...	292	275	94.2	516	455	88.2	85	84	98.8	430	361	84.0

The bacteria-susceptible-virus-susceptible (BSVS) line for the first four generations showed mortalities to *B. enteritidis* increasing from 82.4 per cent to 92.3 per cent (Table V). At that point, two virus-susceptible lines, A and B, were selected. Both, when tested further with *B. enteritidis*, showed approximately 100 per cent mortality through the twelfth generation and both, when tested with virus, showed initial mortalities of about 68 per cent at the sixth generation, increasing to about 95 per cent at the twelfth generation and averaging 88.2 per cent and 84 per cent.

TABLE VI

Immediate Family Histories of Inbred BSVR Lines with Respect to Mortalities Following Test Injections of B. enteritidis or Virus

Line bacteria-susceptible-virus-resistant (BSVR)						
Generation	Progeny tested with <i>B. enteritidis</i>			Progeny tested with virus		
	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead
4	9	9	100			
5	15	15	100			
6	21	21	100	59	1	1.7
7				42	1	2.4
8				33	3	9.1
9	15	14	93.3	70	6	8.6
10	9	8	88.9	19	1	5.3
11	37	35	94.6	46	6	13.0
Totals	106	102	96.2	269	18	6.7

The bacteria-susceptible-virus-resistant line (Table VI), commenced at the fourth generation, showed thereafter through the eleventh generation an average mortality to *B. enteritidis* of 96.2 per cent without significant variation and mortalities to virus from 1.7 per cent to 13.0 per cent, averaging 6.7 per cent of 269 tested mice.

TABLE VII

Immediate Family Histories of Inbred BRVS Line with Respect to Mortalities Following Test Injections of B. enteritidis or Virus

Line bacteria-resistant-virus-susceptible (BRVS)						
Generation	Progeny tested with <i>B. enteritidis</i>			Progeny tested with virus		
	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead
1	4	0	0			
2	16	0	0			
3	22	0	0			
4	82	14	17.1			
5	29	5	17.2	20	19	95
6	43	15	34.9	39	38	97.4
Totals	196	34	17.3	59	57	96.6

The bacteria-resistant-virus-susceptible line (Table VII) showed mortalities to *B. enteritidis* increasing from 0 per cent to 34.9 per cent and averaging 17.3 per cent and mortalities to virus relatively stable at 96.6 per cent.

The three inbred lines have shown percentage mortalities to *B. enteritidis* and virus averaging as follows: BSVS, 95.2 and 86.3 per cent; BSVR, 96.2 and 6.7 per cent; BRVS, 17.3 and 96.6 per cent. High susceptibility proved relatively stable throughout, while high resistance appeared to fluctuate from generation to generation.

The second set of data comprised repeated tests with *B. enteritidis* and virus on divided litters of each line.

TABLE VIII

Mortalities of Inbred Lines Following Injections of B. enteritidis or Encephalitis Virus in Divided Litters

Date	Bacteria-susceptible-virus-susceptible line (BSVS)						Bacteria-susceptible-virus-resistant line (BSVR)						Bacteria-resistant-virus-susceptible line (BRVS)					
	Tests with <i>B. enteritidis</i>			Tests with virus			Tests with <i>B. enteritidis</i>			Tests with virus			Tests with <i>B. enteritidis</i>			Tests with virus		
	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead
1934																		
Nov. 2	19	19	100	10	10	100	19	19	100	10	1	10	7	0	0.0	5	5	100
1935																		
Jan. 29	49	48	98	49	46	93.9	50	47	94	50	8	16	50	8	16	50	46	92
Oct. 1	47	46	97.9	51	50	98	43	41	95.3	40	8	20	58	13	22.4	67	65	97
Nov. 25	12	12	100	15	15	100	12	12	100	22	1	4.5	11	2	18.2	10	9	90
Totals...	127	125	98.4	125	121	96.8	124	119	96.0	122	18	14.8	126	23	18.3	132	125	94.7

Litters aged 5 weeks from each strain were divided each into two approximately equal batches. One batch was given *B. enteritidis per os*; the other encephalitis virus *per nares*. Four such consecutive tests on divided litters are recorded in Table VIII.

The average mortalities (Table VIII) were as follows: BSVS line, *B. enteritidis* 98.4 per cent, virus 96.8 per cent; BSVR line, *B. enteritidis* 96 per cent, virus 14.8 per cent; BRVS line, *B. enteritidis* 18.3 per cent, virus 94.7 per cent. These figures closely approximate the average figures for twelve generations given above (Tables V to VII),

except for a 10 per cent higher level of susceptibility to virus in the BSVS and BSVR lines, and are taken to represent the susceptibilities of the lines used in the cross-breeding experiments which follow.

A final comparison of strains was made with respect to their ability to resist different quantities of the infectious agent. *B. enteritidis* was administered *per os* in doses varying from 5×10^3 to 5×10^5 each to batches of 10 to 20 mice of each strain. Encephalitis virus was instilled intranasally in dilutions of 10^{-1} to 10^{-5} to similar batches. The result was in accord with previous findings that the mice of a given line react similarly to a certain range of dosage of organisms. The susceptible lines showed high mortalities following doses as little as 1/100th of the standard and the resistant lines showed low mortalities following doses 100 times the standard. Consequently the resistant lines, both bacterial and virus, were considered to withstand 1,000 to 10,000 times the dose fatal for susceptibles.

Cross-Breeding Experiments

The three lines were now regarded as sufficiently stable to carry out cross-breeding and backcross experiments for the purpose of analyzing the mechanism of inheritance.¹ The susceptibility level has remained close to 95 per cent, and the resistance level 10 to 20 per cent (Tables V to VIII). Previous cross-breeding of the bacteria-resistant with the white-face susceptible strain (1) had shown resistance to be dominant in the F_1 progeny, and a segregation of resistance and susceptibility in the backcross progeny on the basis of inheritance. The following tests were made on both bacterial factors and virus factors.

Jan. 4, 1935. A. 2 BSVS males were mated to 8 BSVR females in cages containing 1 male to 4 females, respectively. 2 BSVR males were likewise mated to 8 BSVS females. B. Secondly, 2 BSVS males were mated with 8 BRVS females and 2 BRVS males with 8 BSVS females. C. Thirdly, 2 BSVR males were mated with 8 BRVS females and 2 BRVS males with 9 BSVR females.

¹ Variance in mortalities of progeny from each line to *B. enteritidis* and virus infections was tested by the Lexian ratio formula and found to be well within normal expectancy; $P = >0.10$. A similar test on litters from the BSVR \times BRVS cross-mating described below gave the same result.

June 11, 1935. Similar matings were made with A, 2 BSVS males and 8 BSVR females and 2 BSVR males and 8 BSVS females, and with C, 4 BSVR males with 20 BRVS females and 4 BRVS males with 18 BSVR females.

F₁ progeny of these matings, when 4 to 6 weeks of age, were tested with the standard dose of *B. enteritidis*, *per os*, 5×10^6 organisms, or encephalitis virus *per nares*, 0.03 cc. of a 10^{-2} dilution. The litters were divided for the most part, one-half receiving the bacteria, the other half the virus. A few litters were tested with virus alone or with both bacteria and virus in a manner described below.² All methods gave similar results.

The results of tests on a total of 1,448 F₁ progeny comprising 253 litters are summarized in Table IX. F₁ progeny of the A crossing, BS (98 per cent) VS (97 per cent) with BS (96 per cent) VR (15 per cent) lines, would be expected, on the basis of resistance to virus being dominant, to be bacteria-susceptible-virus-resistant. Actually, of 179 tested with *B. enteritidis*, 86.6 per cent succumbed,

² Three methods of double injection were compared: A, giving bacteria and virus at the same time, B, bacteria followed 5 weeks later by virus, and C, virus followed by bacteria. F₁ BRVR mice, whose mortalities to bacteria and virus averaged 26.2 per cent and 16.7 per cent respectively according to the divided litter and single injection tests (Table IX), were treated as follows: A. 20 were given the standard dose of encephalitis virus *per nares*, 0.03 cc. of a 1 to 100 dilution, followed immediately by the standard dose of *B. enteritidis*, *per os*, 5,000,000 bacilli. B. 10 received *B. enteritidis* followed by virus 5 weeks later. C. 10 received virus followed by bacteria 5 weeks later. As controls, 3 BRVS mice were given virus and 3 BSVR were given *B. enteritidis*. All controls succumbed promptly. Of the A batch, 70 per cent succumbed, a percentage more than double that encountered by the single injection method. Of the B mice, 30 per cent succumbed to *B. enteritidis* and 28.6 per cent of the survivors to virus. Of the C mice, 30 per cent succumbed to virus and 14.3 per cent of the survivors to *B. enteritidis*, a result not at too great variance with the 26 per cent virus and 17 per cent *B. enteritidis* mortalities expected.

In a further test, 46 BRVR mice were given C virus followed by *B. enteritidis* with resulting mortalities of 10.9 per cent and 22.0 per cent respectively; 51 were given B *B. enteritidis* followed by virus with resulting mortalities of 29.4 per cent and 8.3 per cent respectively. As controls, 4 BRVS mice were given virus followed by *B. enteritidis* with 75 per cent and 0 per cent mortalities resulting, and 11 *B. enteritidis* followed by virus with 18.2 per cent and 88.9 per cent fatalities. 8 BSVR mice were given virus followed by *B. enteritidis* with 12.5 per cent and 85.7 per cent mortalities, as expected. The B method of giving *B. enteritidis* followed by virus may activate a quiescent bacterial carrier state; both B and C methods introduce a slight selective effect. The C method of virus followed by bacteria, however, has consistently approximated the effects of single injection tests and is now used to test the resistance of selected strains.

TABLE IX

Mortalities of F₁, F₂, and Backcross Progeny Following Test Injections with B. enteritidis and Encephalitis Virus

Date mated	Matings—Cross-breeding	No. litters tested	Total No. progeny	Tests with <i>B. enteritidis</i>			Tests with encephalitis virus		
				No. injected	No. dead	Per cent dead	No. injected	No. dead	Per cent dead
1935									
Jan. 4	A. 2 ♂ BSVS × 8 ♀ BSVR	13	62	14	12	85.7	54	20	37.0
		22	99	36	32	88.9	80	34	42.5
June 11	2 ♂ BSVS × 8 ♀ BSVR	16	86	60	48	80.0	65	12	18.5
		18	107	69	63	91.3	78	22	28.2
Total F ₁ progeny.....		69	354	179	155	86.6	277	88	31.8
Jan. 4	B. 2 ♂ BSVS × 8 ♀ BRVS	34	250	86	14	16.3	173	130	75.1
		25	132	34	12	35.3	99	71	71.7
Total F ₁ progeny.....		59	382	120	26	21.7	272	201	73.9
Jan. 4	C. 2 ♂ BSVR × 8 ♀ BRVS	29	172	67	14	20.9	127	26	20.7
		18	90	44	13	29.5	58	13	22.4
June 11	2 ♂ BRVS × 9 ♀ BSVR	45	243	166	40	24.1	158	20	12.7
		33	207	132	40	30.3	141	22	15.6
Total F ₁ progeny.....		125	712	409	107	26.2	484	81	16.7
Total F ₂ progeny.....		71	265	265	71	26.8	191	67	35.1
Matings—Backcross									
1935									
Sept. 17	A. 2 F ₁ ♂ (BSVR ♂ × BSVS ♀) × 9 ♀ BSVS	5	14	6	4	66.7	14	7	50.0
		8	46	20	19	95.0	46	25	54.3
Total backcross progeny.....		13	60	26	23	88.5	60	32	53.3
Sept. 17	A. 2 F ₁ ♂ (BSVS ♂ × BSVR ♀) × 8 ♀ BSVR	7	27	21	21	100.0	27	4	14.8
		13	70	52	39	75.0	70	14	20.0
Total backcross progeny.....		20	97	73	60	82.2	97	18	18.6
Sept. 17	C. 2 F ₁ ♂ (BSVR ♂ × BRVS ♀) × 8 ♀ BRVS	14	103	54	12	22.2	103	44	42.7
		8	58	33	3	9.1	58	23	39.7
Total backcross progeny.....		22	161	87	15	17.2	161	67	41.6
Sept. 17	C. 2 F ₁ ♂ (BRVS ♂ × BSVR ♀) × 9 ♀ BSVR	6	47	42	20	47.6	47	5	10.6
		12	81	57	29	50.9	81	15	18.5
Total backcross progeny.....		18	128	99	49	49.5	128	20	15.6

and of 277 tested with virus 31.8 per cent succumbed. F_1 progeny of the B crossing, BS (98 per cent) VS (97 per cent) with BR (18 per cent) VS (95 per cent), should be bacteria-resistant and virus-susceptible on the basis of resistance to bacteria being dominant (1). Actually 21.7 per cent of 120 succumbed to *B. enter-*

TABLE X

Comparison of Experimental Data of Cross-Breeding Tests with Results Expected on Basis of Single Factor Type of Inheritance

Symbols	Three tested lines of mice
A = bacteria-resistant (BR) a = bacteria-susceptible (BS)	aa = bacteria-susceptible (BS—98.4 per cent) virus-susceptible (VS—96.8 per cent)
B = virus-resistant (VR) b = virus-susceptible (VS)	aa = bacteria-susceptible (BS—96.0 per cent) virus-resistant (VR—14.8 per cent)
	AA = bacteria-resistant (BR—18.3 per cent) virus-susceptible (VS—94.7 per cent)

First generation matings

Genetic formulae	Expected results	Experimental data (per cent mortality)
1. $\frac{aa}{bb} \times \frac{aa}{BB} = \frac{aa}{bB}$	Bacteria-susceptible Virus-resistant	Bacteria test—86 per cent mortality Virus "—31 " " "
2. $\frac{aa}{bb} \times \frac{AA}{bb} = \frac{aA}{bb}$	Bacteria-resistant Virus-susceptible	Bacteria "—21 " " " Virus "—73.9 " " "
3. $\frac{aa}{BB} \times \frac{AA}{bb} = \frac{Aa}{Bb}$	Bacteria-resistant Virus-resistant	Bacteria "—26.2 " " " Virus "—16.7 " " "

Second generation matings

Genetic formulae	Expected results	Experimental data (per cent mortality)
F_2 from F_1 of 3, $\frac{aA}{Bb} \times \frac{aA}{Bb}$	Bacteria-susceptible—25 per cent Virus-susceptible —25 " "	Bacteria test—26.8 per cent mortality Virus "—35.1 " " "
Backcross F_1 of 1, $\frac{aa}{bB} \times \frac{aa}{bb}$	Bacteria-susceptible—100 " " Virus-susceptible —50 " "	Bacteria "—88.5 " " " Virus "—53.3 " " "
$\frac{aa}{bB} \times \frac{aa}{BB}$	Bacteria-susceptible—100 " " Virus-susceptible —0 " "	Bacteria "—82.3 " " " Virus "—18.6 " " "
Backcross F_1 of 3, $\frac{aA}{Bb} \times \frac{AA}{bb}$	Bacteria-susceptible—0 " " Virus-susceptible —50 " "	Bacteria "—17.2 " " " Virus "—41.6 " " "
$\frac{aA}{Bb} \times \frac{aa}{BB}$	Bacteria-susceptible—50 " " Virus-susceptible —0 " "	Bacteria "—49.5 " " " Virus "—15.6 " " "

itidis and 73.9 per cent of 272 to virus. F_1 progeny of the C crossing, BS (96 per cent) VR (15 per cent) with BR (18 per cent) VS (95 per cent) lines, would be expected to be resistant to both bacteria and virus. On testing, 26.2 per cent of 409 mice given *B. enteritidis* and 16.7 per cent of 484 given virus succumbed.

The tests of F_1 progeny show that susceptibility to *B. enteritidis* and to encephalitis virus is controlled basically by inherited factors, with resistance dominant over susceptibility. Moreover, progeny from a given cross-breeding, A, B, or C, reacted similarly regardless of whether male or female parent carried resistance or susceptibility factors, indicating absence of sex linkage of the inherited factors.

Backcross matings for segregation of resistance factors according to heredity were carried out in the following manner.

Sept. 17, 1935. F_1 progeny from the A mating, having proved susceptible to bacteria and resistant to virus, BS (87 per cent) VR (32 per cent), were backcrossed to both the original bacteria-susceptible-virus-susceptible, BS (98 per cent) VS (97 per cent), and bacteria-susceptible-virus-resistant, BS (96 per cent) VR (15 per cent), lines. In the first instance in which the backcross progeny would be expected on the basis of a simple type of inheritance to be highly susceptible to bacteria and 50 per cent susceptible to virus, 88.5 per cent of 26 tested with *B. enteritidis* and 53.3 per cent of 60 tested with virus succumbed (Tables IX and X). In the second instance in which the backcross progeny should be susceptible to bacteria and resistant to virus, 82.2 per cent of 73 tested with bacteria and 18.6 per cent of 97 tested with virus succumbed.

F_1 progeny from the C mating having proved resistant to both bacteria and virus, BR (26.2 per cent) VR (16.7 per cent), were backcrossed to the original BR (18 per cent) VS (95 per cent) and BS (96 per cent) VR (15 per cent) lines. In the first instance, the backcross progeny would be expected to be resistant to bacteria and 50 per cent susceptible to virus. This expectancy was approximated by actual mortalities of 17.2 per cent of 87 tested with bacteria and 41.6 per cent of 161 tested with virus (Tables IX and X). In the second instance, the backcross progeny should be 50 per cent susceptible to bacteria and resistant to virus. Actually 49.5 per cent of 99 succumbed to *B. enteritidis* and 15.6 per cent of 128 to encephalitis virus.

Finally, F_1 mice derived from the C matings, BSVR \times BRVS, which on testing proved resistant to both bacteria and virus, BR (26.2 per cent) VR (16.7 per cent), were mated brother to sister. The F_2 progeny resulting were tested for the most part with both bacteria and virus by the method described below. Of 265 tested with encephalitis virus, 71 or 26.8 per cent died and of the 191 survivors tested with *B. enteritidis*, 67 or 35.1 per cent died. Similar rates were obtained when batches were injected with one agent only, or when litters were divided and one agent given to one lot, and the other to the remainder.

The results of the cross-breeding tests support the selective breeding data in indicating that resistance to these infections is controlled basically by inherited factors and confirm our previous findings (1)

that the factors are not sex-linked and that resistance is dominant over susceptibility. Besides, they demonstrate that the factors regulating resistance to *B. enteritidis* are not related to those regulating resistance to encephalitis virus. Finally, they indicate that the mechanism of this inheritance may be relatively simple, since the mortality percentages of F_1 , F_2 , and backcross mice approximate for the most part those expected on the basis of two single factor crossings (Table X).

Development of BRVR Line

Throughout the breeding and testing of the three strains for resistance and susceptibility to *B. enteritidis* and encephalitis virus, no evidence of a strain highly resistant to both agents was at hand. BRVR mice were encountered, however, when the BSVR strain was crossed with BRVS (Tables IX and X). Moreover, these doubly resistant F_1 mice, when bred *inter se*, showed an average mortality rate in the neighborhood of that to be expected on the basis of a two single factor type of inheritance. Hence it was assumed that among these F_2 progeny, a pure BRVR strain might be present. To segregate this possible strain from heterozygous BRVR reactors, the following procedure was adopted.

A number of F_2 litters survived the double injection test of *B. enteritidis* followed 5 weeks later by virus. Their parents (F_1) were selected, mated again, and the resulting litters, comprising 42 males and 147 females, siblings of those tested, were mated brother to sister for F_3 progeny to be tested. 122 F_3 litters, totaling 610 individuals, were tested, with an average 30 per cent mortality to *B. enteritidis* and 25 per cent to virus. Certain of these litters survived *in toto* the double injection. The F_2 parents of these were then mated to the original BSVS strain on the supposition that the latter strain was doubly recessive and that the cross-mating would disclose the presence of homozygous F_2 individuals.

These experiments are now in progress and certain lines are emerging which to date are resistant to both *B. enteritidis* and virus.

High Susceptibility of All Selected Strains to Rabies Virus

To louping ill and St. Louis encephalitis viruses, the selected mouse strains react in a consistent manner. To mouse passage rabies virus, however, they all proved highly susceptible (12). The following protocol is illustrative of the reaction.

Batches of twelve of each of the BSVS, BSVR, and BRVS strains were injected as follows: intracerebrally with 0.03 cc. diluted 1 to 100,000 and 1 to 500,000; intralingually with 0.03 cc. diluted 1 to 100 and 1 to 1,000, and into the calf muscle, with 0.03 cc. diluted 1 to 10 and 1 to 30. Thus, 60 mice of each strain were compared for susceptibility to different doses and by different routes. The rabies strain employed, No. 1, passage 70, was obtained from the hippocampal lobe of a dog suffering with rabies and was subsequently passed through 70 mice by intracerebral injection of brain tissue.

All of the 12 BSVS and 11 of the 12 BSVR mice succumbed following the intracerebral injection of the 1 to 100,000 dilution and following the smaller intracerebral dose of 1 to 500,000 dilution, 8 of 12 BSVS and 5 of 11 BSVR mice died. The lingual injection of 1 to 100 dilution of virus was uniformly fatal to all; 1 to 1,000 killed 7 of 12 BSVS and all BSVR mice. Injection into the calf muscle of 1 to 10 dilution was fatal to all alike; less virus, 1 to 30 dilution, killed 9 of 12 BSVS and 11 of 12 BSVR mice.

No significant differences in mortality between strains in this or other tests were apparent following injection of moderate or minimum doses of virus by natural or artificial routes.

DISCUSSION

The groundwork of experimental knowledge of the inheritance of resistance to infectious disease was laid by workers with plant diseases. Biffen, in 1905 (13 a), crossbred strains of wheat resistant to yellow rust with strains susceptible and by testing F_1 and F_2 progeny under field conditions, demonstrated its dependence upon a single factor type of inheritance with susceptibility dominant. His homozygous strains were not completely resistant or susceptible, respectively, but mainly of high or low resistance. He noted that modifications in amount of available nitrogen increased the susceptibility of genetically resistant individuals. Burkholder, in 1918 (13 b), and McRostie, in 1919 (13 c), studying the inheritance of resistance of beans to anthracnose, employed non-infected greenhouse stock and controlled dosage by inoculating each individual in the greenhouse with a similar amount of the more pure strain of the infecting agent. Each found resistance inherited and based upon a single factor mechanism with resistance dominant. Many plant infections have now been studied with techniques aiming at once at naturalness of mode of infection consistent with adequate control. Results are compared with those of a field test. Resistance is found to be inherited in some instances

on a multiple and in some on a single factor basis with resistance dominant (resistance of wheat to (a) stem rust, (b) leaf rust, (c) bunt; resistance of (d) barley to rusty blotch, (e) cabbage to yellows, (f) maize to rust, (h) oats to loose smut) more frequently than susceptibility (13 d to k). Inborn resistance to one infection generally proved independent of resistance to another. No anatomical or physical mechanism has thus far been proved causally related to resistance or susceptibility.

Workers on infectious diseases of animals have paid little attention until recently to the possible regulation of resistance by inborn factors and to the differentiation of individuals according to these factors. Common practice shows that if a batch of animals is given an injection of a virulent agent by some artificial route, the great majority succumb within a few hours. Again, if less virulent agents, smaller doses, or more natural routes of infection are used, a percentage of a random batch of individuals may survive, but if the test is run in duplicate or repeated, the percentage of survivors varies in a random manner. Greenwood and Topley have experience with this sort of result and attribute differences in survival rate and in fate of individuals to uncontrolled errors of technique (14).

An experimental attack on the question in fowl and rodents was undertaken by Frateur in 1924 (3), Roberts and Card in 1926 (4), Lambert and Knox in 1928 (5), Irwin in 1929 (6), Schott in 1932 (7), and Gowen and Schott in 1933 (8). All bred for resistance from survivors of a highly artificial infection and noted a progressively declining mortality. They crossed the selected survivors with either the original unselected stock or with a susceptible strain and tested F_1 , F_2 , and backcross progeny. Frateur (3) and Gowen and Schott (8) interpreted their figures as suggesting a single factor type of Mendelian inheritance with resistance dominant; the remainder accounted for their data on a multiple factor basis. These workers employed materials and techniques, however, which render their results difficult of interpretation from an infectious disease point of view. In the first place, the test infection is suspected of persisting in their stock since survivors are used for breeding. The presence of the infection involves risks of dam and sire infecting each other and of a part of the litter dying from the infection, leaving survivors for later tests which

may prove resistant (*a*) because of their selection through previous infection or because possessed of (*b*) an active or (*c*) a passive immunity. Irwin (6) and Gowen and Schott (8) especially have endeavored to minimize the significance of a transfer of passive immunity in influencing their data, but the probability remains that the persistent infection is a factor in enhancing the resistance of their test progeny. In the second place, resistance has been tested to highly artificial infections,—intraperitoneal injections of large doses, bacteria of low natural infectivity,—without determining experimentally whether such tests are in fact a measure of the resistance of the individual to the infection in nature. Indeed, experiment has often shown the contrary to be the case in that resistance of animals to bacterial infections differs according to the portal of entry employed, and animals susceptible by an artificial route are not necessarily as susceptible by a natural route, and *vice versa*. In the present state of knowledge, therefore, it is important for the worker to indicate clearly the type of resistance he is studying and determine in each instance the degree to which this resistance is a measure of resistance in nature.

Our investigations in inborn resistance commenced (1923 (9)) when it was found that batches of animals bred in the laboratory in an effort to control all possible environmental variables, if exposed to infectious agents in a way simulating nature, differed from batches of uncontrolled mice of the sort discussed by Greenwood and Topley (14) in responding as a group in a relatively predictable manner. Moreover, the survival of some individuals, as contrasted with the death of others under apparently similar and controlled conditions, took on a possible significance. Although the reaction of a given individual of the group could not be predicted, the differences in individual response were regarded as possibly due not to technical irregularities, but to differences in their degree of inborn resistance.

This idea was supported by experiments showing that certain strains of mice suffered consistently higher mortalities than others following *per os* instillation of mouse typhoid bacilli (15) and also following exposure to a naturally spreading herd infection (16). Selective breeding experiments (17) showed that progeny of individuals surviving a *per os* instillation of mouse typhoid bacilli suffered less mortality, and progeny of individuals succumbing early to the infec-

tion suffered greater mortalities following the test infection than the unselected controls. In these tests, however, the original population was too small to insure the selection of individuals with the widest possible differences in inborn resistance characteristic of the strain, and was too small to insure that the selected lines would provide a sufficient number of fertile dams. Then too, the resistant lines were bred from survivors, and the bread and milk diet produced mice whose mortalities fluctuated with season. The findings, however, in spite of their limitations, pointed consistently toward the presence of innate differences in resistance. Consequently, the breeding experiments were repeated (1) with an original population of 600 to cover the susceptibility range of the strain and allow for selection of optimum breeders with progeny exhibiting the desired maximum and minimum mortalities. Moreover, a diet was used which gave relatively stable mortality percentages in unselected controls. Here, although an error was committed in making the first selection for resistants from survivors rather than from the unexposed progeny of mice which were later proved survivors, these selected survivors were proved free of infection before introducing them into the colony and thereafter selections were made from unexposed sibling litters. The colony has remained free of infection from the outset.

The present experiments in their entirety demonstrate that the individual constituents of any sizable population of mice in which environmental variables have been controlled as far as possible differ widely in their innate resistance to infectious agents. This difference is of the order of 1,000 lethal doses or of mortalities to a standard dose of 95 per cent as contrasted with 15 per cent. These innate differences in the resistance of individual mice were brought out by the progeny test and by the development of lines from certain individuals by selective breeding. This procedure segregated at the outset individuals whose resistance was maximum or minimum, respectively, remaining unchanged for twelve generations. The resistance of the majority, however, was intermediate, increasing or decreasing on repeated selection from generation to generation. Crossing the initially highly susceptible and resistant lines and testing F_1 , F_2 , and backcross progeny resulted in percentage mortalities in the neighborhood of those expected on the basis of a single factor type of Men-

delian inheritance for resistance to *B. enteritidis* and to encephalitis virus. Resistance proved dominant in each instance. Moreover, the histories of the direct descendants in each line (Tables V to VII) support the theory of a single factor type of inheritance, since mortalities in succeeding generations showed mainly no definite progress with selection but proved relatively stable. Consequently, we regard the above as evidence of a single main factor type of inheritance with possibly a number of small modifiers.

Individuals inherently resistant or susceptible to one infectious or toxic agent may or may not prove likewise resistant or susceptible to another. Previously we noted that survivors of mouse typhoid or individuals fed on McCollum ration were relatively resistant not only to a subsequent injection of an antigenically different strain of mouse typhoid but to HgCl_2 (18, 19). This indicated to us that resistance was conditioned not only by specific immunity but by non-specific factors as well. The association of resistance to mouse typhoid and to HgCl_2 was considered not as necessary nor as an indication of a pan-resistance, as Hill (2) has inferred. Rather the parallel was one of chance. This supposition is borne out by the present studies in which from hybrid stock, lines were segregated with various combinations of resistance and susceptibility, namely, BSVS line, susceptible to two enteric and three respiratory tract bacterial and three virus infections, BRVR line, resistant to all save rabies, BSVR line, susceptible to the bacterial and resistant to two of the three tested virus infections, and the BRVS line, resistant to the bacterial and susceptible to the virus infections. And finally, the cross-breeding of these strains and testing of progeny brought out the independence of genetic factors governing resistance to *B. enteritidis* and encephalitis virus, respectively. It follows that the amount of inherent resistance displayed by an individual to an infectious agent cannot be taken without experiment as a measure of its resistance to another.

The effect of unforeseen environmental variables on the manifestation of genetic factors is disturbing in carrying out this sort of experiment. Control measures are frequently inadequate to prevent variations in results such as the sudden increase of 15 to 20 per cent in mortality percentages in all lines recorded in the present experiments.

The expression of genetic factors is conditioned by the expression of somatic factors and the experiments can achieve at best but a measure of the summation effect of both under conditions in which the environmental ones have been controlled as far as possible.

In concluding this portion of the discussion, we point out again the close parallelism between our findings and those of workers on plant diseases.

The thesis of variability of host resistance and its regulation by inborn and environmental factors has both particular and general bearing upon experimentation in infectious disease. The particular effect of innate factors on type of clinical disease and tissue changes is exemplified in studies on susceptible and resistant mice following oral administration of *B. enteritidis* (1), and nasal administration of pneumococci (20) and encephalitis virus (21). Again the rôle of innate resistance factors is being investigated in experimental epidemiology, for example, in the matter of determining the status of survivors of an epidemic. Are they inherently resistant at the outset and spared from the ravages of the epidemic agent, or are they differentiated only by the chance exposure to subinfectious doses which have immunized them, or do both processes participate?

Greenwood and Topley have studied the question in herds of infected mice to which normal animals were added daily (14). Data on cage age of mice at death were set out in the form of life tables, but their analyses indicate merely that in mouse populations of this sort as well as in human populations, the mode of action of host factors remains in the field of conjecture. The lack of control in these experiments merely complicates the simple problem long familiar to immunologists, namely, are survivors of a relatively natural test infection resistant to a subsequent exposure because of innate resistance or acquired specific immunity factors, or both? The question in this simple test or in the herds of English mice cannot be answered until the resistance of individuals in the herd at the beginning of the experiment is measurable. One experiment fulfilling this requirement has been reported (1). In each of thirty-six tests, 5 mice of known 37 per cent mortality following standard test were given the stomachal instillation of *B. enteritidis* and placed in a cage with 5 or 3 mice 85 per cent susceptible and with a similar number 15 per cent susceptible.

The infection was allowed to spread from the infected to the two classes of contacts hitherto unexposed. Of the 194 susceptibles, 70 per cent succumbed, as contrasted with 12 per cent of resistants. This left the surviving populations at the close of the epidemic composed of 70 per cent of individuals known at the outset to be innately highly resistant. This indicates clearly that survivors of this type of experiment are largely selected resistants. The remaining question of what sort of mice are subclinically immunized and how readily is subclinical immunization accomplished is now under study. At present we find immunization difficult under natural conditions with susceptibles and readily accomplished with resistants.

In general, the concept of control of host variables enforces a conservative attitude in judging seemingly contradictory results of different workers unless their test animals are comparable in all respects. And finally, standardized animals, like pure reagents in chemistry, should provide a means of elucidating many of the quantitative problems in infectious disease.

CONCLUSIONS

Under the conditions specified, there may be selected promptly from a hybrid stock of mice, of which 40 to 50 per cent die following a standard dose of *B. enteritidis* or St. Louis encephalitis virus, lines in which as high as 95 per cent and as low as 15 per cent succumb. Three lines,—one bacteria-susceptible-virus-susceptible, one bacteria-susceptible-virus-resistant, and one bacteria-resistant-virus-susceptible,—are regarded as remaining relatively stable after approximately twelve generations of selection and brother to sister or line inbreeding.

Crossing susceptible with resistant lines and testing F_1 , F_2 , F_3 , and backcross progeny resulted in mortality percentages in the neighborhood of those expected on the basis that resistance to *B. enteritidis* and to encephalitis virus is each inherited independently on a single factor basis with resistance dominant over susceptibility.

A bacteria-resistant-virus-resistant line is being developed from a cross between bacteria-susceptible-virus-resistant and bacteria-resistant-virus-susceptible lines.

All selected lines proved uniformly susceptible to a strain of mouse passage rabies virus.

Miss Alfhild Johnson assisted with the technical part of these experiments.

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EXPERIMENTAL PRODUCTION OF HEMORRHAGE AND VASCULAR LESIONS IN LYMPH NODES: AN EXTENSION OF THE SHWARTZMAN PHENOMENON

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PLATE 7

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Since the description by Shwartzman (1) of a phenomenon of local skin reactivity to bacterial filtrates, hemorrhagic lesions have been produced in many organs by variations of this reaction. Following the intravenous injection of certain bacterial filtrates, the Shwartzman phenomenon (2) has been demonstrated in various tissues previously prepared by the local injection of these filtrates or by the local inoculation of certain viruses. Despite the importance of the lymphatic system in the absorption of material from an injected site (Hudack and McMaster (3)), no detailed study has been made of its relation to the Shwartzman phenomenon.

Shwartzman (4) was able to elicit a phenomenon of local reactivity in the lungs and kidneys. The reaction has also been produced in the stomach (Karsner, Ecker and Jackson (5)), joints (Moritz and Morley (6)), testicle, peritoneum and intestines (Gratia and Linz (7)) and brain (Bock (8)). It has also been seen in sarcomatous tissue (Gratia and Linz (9)), Shwartzman and Michailovsky (10)). Bordet (11) noted congestion and hemorrhage following an intraperitoneal inoculation with a sterilized *Bacillus coli* suspension in the mesenteric lymph nodes of guinea pigs previously vaccinated with BCG by this route. It is noteworthy that an intraperitoneal injection may be substituted for the intravenous in the production of the Shwartzman reaction (Frisch (12)). Gratia and Linz (13) observed that after an intravenous injection of *B. coli* filtrate, rabbits with an active vaccine virus infection showed hemorrhage in the regional lymph nodes as well as at the site of infection. In neither the experiments of Bordet nor those of Gratia and Linz were histological studies mentioned regarding the nature of the lesions in the lymph nodes or their possible association with the Shwartzman phenomenon.

The purpose of the work described in this paper was, first, to elicit the Shwartzman phenomenon in lymph nodes by preparation with bacterial filtrates through the lymphatics, and second, to study the relation of the lymphatic system to the phenomenon.

Anatomy of the Regional Lymphatic System.—In the inguinal region along the course of the circumflex abdominal vessels is a subcutaneous lymph node which drains the skin and subcutaneous tissues of the lower part of the abdomen and the medial surface of the thigh. This node is called the inguinal lymph node. An efferent lymphatic from this node accompanies the circumflex abdominal vein and artery and then the femoral (crural) vessels to terminate in the common iliac node of the same side.¹ These common iliac nodes are situated on the posterolateral surface of the common iliac arteries. They are associated with the hypogastric node by fine anastomosing lymphatics. This latter node is found in the hollow of the sacrum closely applied to the anterior aspect of the hypogastric veins. Thus, a 1 per cent aqueous solution of trypan blue injected into the skin of the lower part of the abdomen or the medial surface of the thigh will go to the corresponding inguinal node and subsequently be found coloring hypogastric and homolateral common iliac nodes. Similarly, dye injected into the femoral or circumflex abdominal lymphatics will go directly to the latter nodes. Intradermal injections made more cephalad will drain also to subcutaneous nodes posterior to the lateral thoracic vessels in the pectoral region or to the axillary node which lies in the axilla medial to the axillary vein. If intradermal injections of dye are made near the midline of the belly they may drain to the lymph nodes of the opposite side. The ilio-lumbar lymph node is concerned with the lymphatic drainage of the skin of the lateral surface of the thigh and of the lower back. It is a subcutaneous node along the course of the ilio-lumbar vessels, slightly posterior to the anterior superior spine of the ilium.

The Shwartzman Phenomenon in Lymph Nodes

Procedures.—With aseptic precautions, under ether anesthesia, an incision was made in the skin of the groin of normal rabbits weighing 2000–4000 gm., and not over 14 months old. Using a short bevel No. 26 gauge intradermal needle, meningococcus (15) or *B. typhosus* (16) culture filtrate was injected into the circumflex abdominal lymphatic in the direction of the femoral vessels. The filtrates were given in dilutions varying between 1:30 and undiluted and in amounts of 0.1–

¹ The terminology used in this description corresponds generally to that given by Krause (14). However, the group of lymph nodes which he designated as hypogastric is here subdivided into hypogastric and right and left common iliac nodes. The term ilio-lumbar is preferred for the subcutaneous lymph node found along the course of the ilio-lumbar vein and artery because its location and the area it drains is different from that of the node called inguinal in the present paper.

0.3 cc. (Table I). Care was taken not to traumatize the lymphatic except during the actual injection of the filtrate. When difficulty was experienced in injecting the fluid, the operation was immediately performed upon the opposite side. After the filtrate was injected into the lymphatic, the skin incision was closed with silk.

24 hours after the initial injection, the animals were given an intravenous dose of the filtrate in a dilution equivalent to 5 to 25 reacting units, per kilo of body weight (17) (Table I).

The animals were killed 4 to 6 hours after the intravenous injection and autopsied. The common iliac and hypogastric nodes and usually the mesenteric, ilio-lumbar, pectoral and axillary nodes, were inspected and taken for study. As controls on the combined injections, an equal number of rabbits were killed and autopsied 24 to 30 hours after the intralymphatic injection of filtrate without the administration of intravenous filtrate. Comparable lymph nodes from these animals were removed. The animals were killed by continuous administration of ether with ready access of air.

The lymph nodes were fixed in Zenker-formol solution or 10 per cent formalin and routine hematoxylin-eosin stains were made. Mallory's aniline blue stain, Weigert's fibrin stain, Foot and Foot's silver impregnation for reticulum (18) and Verhoeff's elastic tissue stain counterstained with van Gieson's picrofuchsin solution were also used on representative sections.

The *B. typhosus* filtrates² used in this work were filtrates of saline washings of agar cultures made from strain T₁ according to the method described by Schwartzman (16). The meningococcus filtrates were prepared from a group II strain 383 received from Dr. Schwartzman. These were also made from saline washings (15). The number of reacting units per cubic centimeter of the filtrates were determined in his laboratory (17).

Effect of Combined Intralymphatic and Intravenous Injections of Bacterial Filtrates (Group I).—The results of experiments involving the use of meningococcus and *B. typhosus* filtrates were in general quite similar and will, therefore, be considered together. They are summarized in Table I, groups IA and IB.

At the time of autopsy, the incisions in the groin through which the intralymphatic injections had been made, were clean; and when they were reopened, no significant hemorrhage was found in subcutaneous tissues of this region. The common iliac and the hypogastric nodes were prominent, being swollen and generally of a deep reddish purple color. They occasionally showed petechiae scattered over their surfaces. In rabbits 5 and 9 the hemorrhagic appearance of the common iliac nodes was limited to those on the side injected. The inguinal and ilio-lumbar nodes, despite their proximity to the site of manipulation, were hemor-

² The *B. typhosus* filtrates were all obtained through the kindness of Dr. Gregory Schwartzman.

TABLE I

Effect on Lymph Nodes of Injection of Bacterial Filtrates into Afferent Lymphatics with and without Subsequent Intravenous Injection of Filtrate

Group	Rabbit No.	Intralymphatic injection			Intravenous injection (1 cc. per kilo)			Results in common iliac and hypogastric nodes		Remarks
		Material	Dilution	Dose cc.	Material	Dilution	Reacting units per cc.	Hemorrhage	Thromboses	
I A	1	Mgo. 383*	Undiluted	0.25	Mgo. 383	1:10	20	Present	Absent	Rabbit 1 dead, 45 min. after intravenous injection
	2	"	"	0.2	"	1:40	5	"	Present	
	3	"	"	0.15	"	1:20	10	"	"	
	4	"	"	0.15	"	1:40	5	"	"	
I B	5	B.TyT _L † T.1943	1:30	0.1	B.TyT _L T.1943	1:8	25	"	"	
	6	"	1:30	0.1	"	1:8	25	"	"	
	7	"	1:30	0.1	"	1:8	25	"	"	
	8	"	1:30	0.1	"	1:8	25	"	"	
	9	"	1:30	0.1	"	1:8	25	"	"	
	10	"	1:30	0.1	"	1:8	25	"	"	
	11	"	1:30	0.1	"	1:8	25	"	"	

II A	12	Mgo. 383	Undiluted	0.3	None given		Present in hypogastric on section only; absent in iliac	Absent	Hemorrhage considered traumatic
	13	"	"	0.25	"		Absent	"	
	14	"	"	0.25	"		"	"	
	15	"	"	0.25	"		"	"	
II B	16	B.TyT ₂ T.1943	1:5	0.1	"		Microscopically in hypogastric only; absent in iliac	"	Node traumatized in removal
	17	"	1:5	0.1	"		Absent	"	
	18	"	1:5	0.1	"		"	"	
	19	"	1:5	0.1	"		"	"	
	20	"	1:5	0.1	"		"	"	
	21	"	1:5	0.1	"		"	"	

* Abbreviation Mgo. 383 means filtrate of meningococcus strain 383 culture.

† In this and in Table II abbreviation B.TyT₂ means filtrate of *B. typhosus* strain T cultures.

rhagic in the gross in only three instances (rabbits 7, 6, 11). Lymph nodes not directly associated with the site of injection, were normal on gross examination. The rabbits showed no pathological changes other than those noted.

Microscopic examination of the iliac and hypogastric lymph nodes of the rabbits in this series, whether injected with meningococcus or with *B. typhosus* filtrate, generally revealed hemorrhage into the lymphoid tissue. In many instances this hemorrhage was of such extent as to destroy or completely mask the normal architectural relationships. Accompanying the hemorrhage, except in rabbit 1 which died shortly after the intravenous injection, were conspicuous changes in the smaller blood vessels. Capillaries and venules were greatly dilated. Some were markedly engorged; others were partly or completely occluded by thrombus formations (Fig. 1). These thrombi consisted of platelets and eosinophilic fibrillar networks in which were enmeshed many polymorphonuclear leucocytes. With Weigert's fibrin stain they had the appearance characteristic of fibrin. The endothelium of many of the veins and capillaries was much swollen, and projected into the lumina of the vessels. The nuclei of the endothelial cells did not appear to have the normal chromatin content, only the nuclear membrane taking the stain. The thrombi were not infrequently found adherent to the swollen endothelial cells. The walls of some of the arterioles from two of the animals injected with meningococcus filtrate were swollen and had a hyaline appearance. The endothelial cells were shrunken and necrotic (Fig. 2). There was some edema of the perivascular tissue about these arteriolar lesions, but cellular infiltrations were not prominent. Such arteriolar changes were not seen in nodes from other rabbits of group I. No thrombi were seen in any of the larger arteries. Elastic tissue stains (Verhoeff-van Gieson) showed no change in the elastica of the arteries and veins of the nodes or in the perilymphnodular tissue. Disruption of the lymphoid reticulum was demonstrated by Foot and Foot's silver impregnation. Slight to moderate diffuse polymorphonuclear infiltration accompanied the hemorrhage and vascular lesions. Macrophages were plentiful in the lymph sinuses and were filled with red blood cells. The pathological changes which have been described were limited to lymph nodes receiving the direct effect of the injected filtrates.

Effect of Single Intralymphatic Injections of Bacterial Filtrates (Group II).—Rabbits killed 24 to 30 hours after a single intralymphatic injection of meningococcus or *B. typhosus* filtrate, in contrast to those examined following combined intralymphatic intravenous injections, presented little gross pathological change in the lymph nodes that received the filtrates.

None of the common iliac or hypogastric lymph nodes from these rabbits showed any hemorrhages on their surfaces (Table I, groups II A and II B). They appeared normal in size and color. One exception was noted: the hypogastric

node from rabbit 12 appeared congested, and when sectioned presented a sizeable area of hemorrhage in its medullary portion. Mesenteric nodes and those more superficially situated, such as the axillary and iliolumbar nodes, were in all cases negative.

On histological examination the lymph nodes receiving the injected bacterial filtrates were generally slightly edematous and presented a variable polymorphonuclear infiltration. The polymorphonuclear leucocytes were often seen massed in the intermediate lymph sinuses; the follicles generally showed little or no reaction. Macrophages were found in increased numbers in the lymph sinuses, and frequently were filled with masses of cellular debris.

Histologically common iliac nodes from rabbits in these control experiments showed no hemorrhages, vascular alterations or thrombi. Of the hypogastric nodes, only two showed any hemorrhage into the lymphoid tissue.

One was from rabbit 12, injected with meningococcus filtrate, and has already been noted in the macroscopic description. This node presented a large hemorrhage in its medullary portion which was surrounded by a well defined zone of polymorphonuclear leucocytes. It was perhaps traumatic in origin and caused by the sudden introduction of a relatively large amount of fluid (0.3 cc. of filtrate) during the intralymphatic injection. There was blood in the lymph sinuses in this region, but the veins and capillaries were not congested or thrombosed. There were no arteriolar lesions. The second hypogastric lymph node with hemorrhage was removed from rabbit 16 which had been injected with *B. typhosus* filtrate diluted 1:30. This node showed no accentuation of the polymorphonuclear infiltration of the lymph sinuses in the regions of hemorrhage. There were no thromboses in this or other hypogastric nodes of this series. The hemorrhages in this node were also considered traumatic, caused at the time of its removal by considerable bleeding resulting from an accidental cutting of the vena cava.

Lymph nodes unrelated to the injected site, such as the mesenteric nodes or subcutaneous nodes in these control rabbits, were negative in all cases for hemorrhage and vascular lesions.

Effect of Single Intravenous Injections of Bacterial Filtrates on the Lymph Nodes.

—Three stock rabbits were given single intravenous injections of meningococcus culture filtrate 383 in doses of 10 reacting units per kilo. Inguinal, iliolumbar, pectoral, axillary, iliac and hypogastric lymph nodes were all entirely negative on both gross and microscopic examination. Further experiments along these lines were not done, inasmuch as Apitz (19, 20), Freund (21) and Gerber (22) have demonstrated that single intravenous injections of bacterial filtrates, even in lethal doses, do not produce hemorrhages or thromboses in the lymph nodes of normal rabbits.

The experiments that have been described indicate that following an intravenous injection of bacterial filtrate it is possible to elicit the Shwartzman phenomenon in lymph nodes of rabbits prepared by an injection of the filtrate into the afferent lymphatics. Single intralymphatic or single intravenous injections of the filtrates are ineffective in evoking such changes. The state of local reactivity in the lymph nodes is limited to those nodes that receive the direct effect of the filtrates.

Effect on the Lymph Nodes of the Shwartzman Phenomenon in the Skin

In order to study the effect of the Shwartzman phenomenon in the skin on the lymphatic system, an intradermal preparatory injection as originally described by Shwartzman (1) was used. It is well known that materials injected intradermally enter the cutaneous lymphatics (3) and an opportunity is offered thereby to avoid the trauma necessarily incidental to dissection and direct injection of the lymphatics.

Healthy stock rabbits about 2000 gm. in weight and having no visible scratches or infections of the skin were used. The hair of the abdomen and groin was clipped 1 or more days before the experiments.

The rabbits were each injected intradermally with 0.25 cc. *B. typhosus* culture filtrate. 24 hours later they were given an intravenous injection of the same filtrate. The concentration of the intradermal injection varied between a dilution of 1:30 and undiluted; the intravenous dose was 1 cc. per kilo of a dilution equivalent to 25 to 40 reacting units per cc. (Table II, group III). Control rabbits received an intradermal but no intravenous injection (group IV).

The preparatory intradermal injection was usually placed in one of the lower quadrants of the abdomen. In rabbits 24, 25 and 50 (Table II), it was made on the medial aspect of the left thigh.

The site of the preparatory injection was observed immediately before the intravenous injection and again 4 to 5 hours after it. The animals were killed 4 to 6 hours after the intravenous dose, and in the case of controls 24 to 30 hours after the intradermal injection. The only exceptions were rabbits 45, 46 and 47, which were killed 48 hours after the intradermal injection. The regional lymph nodes and those studied following the intralymphatic injections were inspected and examined microscopically.

The lymph nodes were fixed and stained by the methods used on those removed from the animals given intralymphatic injections of bacterial filtrate. The tissue was further examined for fat by Scharlach R.

Effect on Lymph Nodes of Combined Intradermal and Intravenous Injections of B. typhosus Filtrate (Group III).—None of the animals presented any cutaneous hemorrhage before the intravenous injection, and in only three was there some swelling of the prepared skin areas (Table II). Thirteen of 23 rabbits showed definitely positive Shwartzman reactions following the intravenous injections. These varied in intensity and extent from a few isolated punctiform hemorrhages (1+ pf.) to large bluish purple areas (4+) measuring 2 cm. or more in diameter. In all these Shwartzman positive animals, except one (rabbit 28), the regional lymph nodes draining the injected skin sites were hemorrhagic and were larger than those on the uninjected side (Fig. 3). The extent but not the character of the hemorrhage in these nodes appeared to depend on the concentration of the filtrate injected intradermally.

Hemorrhage was sharply limited to the regional lymph nodes which might, however, be several centimeters distant from the prepared site. When the filtrate was introduced into the skin so as to drain into the inguinal nodes, the secondary nodes in the chain, that is, the hypogastric and homolateral common iliac nodes, were usually markedly hemorrhagic.

A lower threshold for the production of the Shwartzman phenomenon in lymph nodes than in the areas of skin which they drain is indicated by the presence of extensive hemorrhagic lesions in the regional lymph nodes of seven out of ten of the rabbits with negative Shwartzman reactions in the skin.

Microscopic study generally revealed bleeding into the substance of the lymph nodes. This might be so extensive as to disturb the entire structure of the node, or of that part of the node involved. In other cases there were multiple hemorrhages into the follicles. The reticulum of these nodes was fragmented in the areas of hemorrhage. There was usually blood in the lymph sinuses. The histiocytes were filled with red blood cells and the red corpuscles were frequently seen sticking to the surface of these cells.

In the hemorrhagic lymph nodes the venules and capillaries in both the cortical and medullary substance were usually congested. Their endothelial cells were greatly swollen and bulged into the lumina of the vessels. Nuclei of these cells took the hematoxylin stain poorly. Scharlach R stain showed that some of the endothelial cells had undergone fatty degeneration. Thrombus formations were found in these vessels. They might completely fill the cross section of the

TABLE II

Effect on Regional Lymph Nodes of Intradermal Injection of *B. typhosus* Filtrate with and without Subsequent Intravenous Injection

Group	Rabbit No.	Preparatory injection (0.25 cc. intradermally)		Reacting injection (1 cc. per kilo intravenously)			Skin reaction		Regional nodes	
		Material	Dilution	Material	Dilution	Re-acting units per cc.	Before intravenous injection	After intravenous injection*	Hemorrhagic in the gross	Thromboses microscopically
III	22	B.TyT _L T.1986	Undiluted	B.TyT _L T.1986	1:14	40	0	4+ (2x2)	Present	Present
	23	"	"	"	1:14	40	0	4+ (2x3)	"	"
	24	"	"	"	1:14	40	(Slight swelling)	0	"	"
	25	"	"	"	1:14	40	"	0	"	"
	26	"	"	"	1:14	40	0	4+ (2x3)	"	"
	27	"	"	"	1:14	40	0	4+ (2x2)	"	"
	28	"	"	"	1:14	40	0	1+ pf.	Present only	Absent
	29	"	1:30	"	1:24	25	0	0	microscopically	Present
	30	"	1:30	"	1:24	25	0	0	Absent	Absent
	31	"	1:30	"	1:24	25	0	2+ (2x2)	Present	Present
	32	"	1:30	"	1:24	25	0	0	Absent	Absent
	33	"	1:30	"	1:20	25	0	0	Present	Present
	34	T.2069	1:30	"	1:20	25	0	0	"	"
	35	"	1:30	"	1:20	25	0	4+ (3x2)	"	"
	36	"	1:30	"	1:20	25	0	4+ (1.5x2)	"	"
	37	"	1:30	"	1:20	25	0	1+ pf.	Absent	"
	38	"	1:30	"	1:20	25	0	0	Present	"
	39	"	1:30	"	1:20	25	0	4+ (1x1)	"	Absent

[illegible]

53	"	"	1:30				
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* The skin reactions after the intravenous injection are graded according to the intensity of the hemorrhage evoked: 0, 1 + pf. (punctiform); 2 + and 4 +. The grading of confluent lesions is followed by their size in centimeters.

vessels or be seen extending from one branch to another. Where they did not completely occlude the vessels, they formed endovascular collars or polypoid projections adherent to the endothelial cells (Fig. 4). Proliferated endothelium was frequently seen covering them. The thrombi consisted of polymorphonuclear leucocytes and platelets enmeshed in delicate networks of fibrin. While generally occurring together, the hemorrhage and thromboses were occasionally seen independently of each other.

The arterioles were constricted rather than dilated and contained little blood. Occasionally there was swelling and edema of their walls.

Regional lymph nodes from rabbits subjected to the Shwartzman reaction, showed an infiltration with polymorphonuclear leucocytes, most marked in the lymph sinuses bordering the medullary cords and in the lymphoid tissue around some of the thrombosed blood vessels. Emigration of leucocytes through the walls of veins and capillaries was occasionally seen. The polymorphonuclear leucocytes were seen in all stages of degeneration, and stained with Scharlach R, these cells, regardless of their location, usually were filled with finely divided globules of fat. Macrophages were very plentiful and contained red cells, vacuoles and much basophilic cellular debris. Phagocytosis of material other than erythrocytes was much more pronounced in rabbits which had been injected intradermally than in those injected by the intralymphatic route. In the follicles with hemorrhage there was often necrosis shown by nuclear fragmentation and disintegration of cells. The perivascular infiltration appeared to be secondary to the vascular damage as there were not infrequently thrombi and other vascular changes described above, with little or no reaction in the perivascular tissue. Further, the amount of hemorrhage in the affected lymph nodes did not bear any constant relation to the severity of inflammation present. Hemorrhage, together with the thromboses, seemed to be a consequence of primary alteration of the blood vessels.

Lymph nodes not draining the regions injected showed none of the pathological changes just described.

Results of Single Intradermal Injections of B. typhosus filtrate (Group IV).—Rabbits killed 24 to 48 hours after an intradermal injection of *B. typhosus* culture filtrate showed no gross evidence of hemorrhage in any of the subcutaneous lymph nodes examined, whether regional or unrelated to the site of injection (Table II, group IV). Deep lymph nodes, such as the common iliac, hypogastric and mesenteric nodes, were normal in appearance in all cases. The injected skin sites never showed hemorrhagic reactions.

Microscopically, regional lymph nodes in rabbits killed 24 to 30 hours after receiving an intradermal injection of *B. typhosus* culture filtrate showed a moderate degree of edema (Fig. 5). There was diffuse infiltration with polymorpho-

nuclear leucocytes throughout the medulla and sinuses, but little if any in the follicles. Many of these leucocytes had pyknotic nuclei. The cellular infiltration was proportional to the concentration of the filtrate injected intradermally. Comparable nodes removed from rabbits killed 48 hours after the intradermal injection of undiluted filtrate still exhibited a considerable number of infiltrating polymorphonuclears. Macrophages which were increased in number in both the follicles and lymph sinuses had phagocytized basophilic cellular debris. There were no phagocytes containing red cells.

Inflammatory reactions were limited to the regional lymph nodes; those not concerned with the drainage of the injected skin site, *e.g.*, the mesenteric nodes and the contralateral superficial and deep nodes, were normal.

No hemorrhage or vascular lesions were seen in the lymph nodes from animals of these control experiments regardless of the concentration of the filtrate used in the intradermal injections.

DISCUSSION

The pathological changes in the Shwartzman phenomenon in the skin and other tissues have been studied by Apitz (23), Moritz and Morley (6), Karsner and Moritz (24) and Gerber (25). These authors all agree that varying degrees of inflammation occur following the preparatory injection with the bacterial filtrate, and that hemorrhage and thrombosis are produced subsequent to the reacting injection. In the experiments just described such changes have also been noted. Karsner and Moritz, however, observed hemorrhage and thrombosis occurring with the initial injection alone and consider the essential difference between this picture and that following the intravenous injection to be a quantitative one. In the present study, hemorrhage and thrombosis have not been seen in the lymph nodes of rabbits which were subjected only to the preparatory injection. The results are, rather, in accord with those of Apitz and of Gerber, who state that in their experiments, the preparatory injection evokes an acute edematous leucocytic reaction without evidence of vascular damage, hemorrhage or thrombosis. In the lymph nodes, as in the skin (Gerber), the amount of inflammation produced following the preparatory injection is proportional to the concentration of filtrate. Further, thrombosis and hemorrhage following the reacting dose are not dependent on the amount of previously existing inflammation. Gerber finds that in the skin, vascular dilatation, congestion and hemorrhage following the reacting injection precede thrombosis. In the

present study thrombosis has been seen after the intravenous injection without congestion and hemorrhage. With the Shwartzman phenomenon there were swelling and fatty degeneration of endothelial cells of thrombosed blood vessels of lymph nodes. Apitz (19) and Gerber noted no endothelial alterations at the point of attachment of thrombi.

CONCLUSIONS

Characteristic changes are produced in the lymph nodes of rabbits following the intravenous injection of certain bacterial filtrates administered 24 hours after either an intralymphatic or an intradermal injection of the same filtrate. These changes are limited to the nodes served by the lymphatic injected or to those furnishing the lymphatic drainage for the injected skin site. By either method the initial or preparatory injection of filtrate reaches the lymph nodes through one or more of its afferent lymphatics, and similar lesions are produced in the nodes.

The lesions consist of hemorrhages recognizable by gross and microscopic examination. The capillaries and veins are congested and thrombosed. Their endothelial cells are swollen. Arterioles are generally little affected. Though hemorrhages and thromboses are usually seen together in the nodes, they have been observed occurring independently. They are both probably secondary to endothelial changes. The lesions are not dependent on the amount of preexisting inflammation in the nodes.

Endothelial changes, hemorrhages and thromboses were usually noted in the regional nodes when positive Shwartzman reactions had been elicited in prepared skin by intravenous injection of the bacterial filtrate. However, these lesions in many instances were observed under similar conditions in these nodes even when the Shwartzman reaction in the skin was negative. It appears that lymph nodes are more susceptible to the production of the Shwartzman phenomenon than the skin sites which they drain.

A single intralymphatic or intradermal injection of the bacterial filtrates used in this study, even in high concentrations, does not produce in adjacent lymph nodes the characteristic changes noted

when this preparatory injection is followed by a subsequent intravenous injection of the filtrate.

Single intravenous injections also are not productive of hemorrhage and thrombosis in lymph nodes.

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EXPLANATION OF PLATE 7

Dilutions and amounts of preparatory and reacting injections are given in Tables I and II.

FIG. 1. Section of hypogastric lymph node from rabbit 7, 6 hours after an intravenous injection of filtrate from culture of *B. typhosus* given following a preparatory injection of the same filtrate into the afferent lymphatic. The dilated veins are partially thrombosed. Macrophages containing red blood cells are in the lymph sinuses. Hematoxylin and eosin. $\times 160$.

FIG. 2. Arteriole in hypogastric node of rabbit 4, showing swelling and hyaline degeneration of the wall and necrosis of the endothelial cells. There is perivascular edema but no cellular infiltration. The animal was killed 6 hours after an intravenous injection of filtrate of meningococcus culture administered 24 hours subsequent to an injection of this filtrate into the circumflex abdominal lymphatic. Hematoxylin and eosin. $\times 600$.

FIG. 3. Sections of right (R) and left (L) pectoral nodes from rabbit 44. The animal was given an intravenous injection of filtrate from a culture of *B. typhosus* 24 hours after a preparatory injection of the same filtrate into the skin of the right side of the abdomen. The right pectoral node is swollen, congested and contains a large area of hemorrhage. The left pectoral node is normal in appearance.

FIG. 4. Section of vein in left common iliac node from rabbit 24, which received an intravenous injection of filtrate from a culture of *B. typhosus* 24 hours after a preparatory injection of the filtrate into the skin of the medial surface of the left thigh. The vein contains a thrombus adherent to endothelial cells which are swollen and take the nuclear stain lightly. Note there is no perivascular cellular infiltration. The Schwartzman reaction was negative at the injected skin site. Hematoxylin and eosin. $\times 650$.

FIG. 5. Regional inguinal lymph node from rabbit 52 removed 24 hours after an intradermal injection of filtrate of culture of *B. typhosus* into the right lower quadrant of the abdomen. The section shows edema of the node. Blood vessels are empty and not dilated or thrombosed. Compare with Fig. 1. Hematoxylin and eosin. $\times 160$.



(Koplik: Hemorrhage and vascular lesions in lymph nodes)

THE RAPID INVASION OF THE BODY THROUGH THE OLFACTORY MUCOSA

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PLATES 8 TO 10

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Clark (1) in 1929 described the introduction of Prussian blue into the nasal cavities of rabbits to determine the routes by which infectious agents pass from these cavities to the brain. At the earliest time of study, 1 hour after nasal inoculation of the pigment, granules were found between the cells of the olfactory mucosa, but not the respiratory, in the submucosa, the lymphatics and venules, in the perineural sheaths and between the fibers of the olfactory nerve, but not the trigeminal, and within the subarachnoid space about the olfactory bulbs. He demonstrated that the perineural spaces of the olfactory nerve are continuous with the subarachnoid space and considered that the granules had reached the olfactory bulbs mainly by passage along the perineural spaces. To account for this migration he postulated a centripetal current in these spaces. He found no evidence of direct communication between the perineural or subarachnoid spaces and the nasal lymphatics and did not determine the method by which the pigment penetrates the mucosa nor the shortest interval in which granules can reach the intracranial cavity.

These essential findings have been confirmed by Olitsky and Cox (2) in experiments with mice. Moreover we (3, 4) have shown that pneumococci instilled intranasally in mice pass rapidly through the mucosal surfaces, including those of the nose, to reach the peripheral blood within 2 minutes. We also, in repeating the tests with Prussian blue in mice, have found (5) that absorption is very rapid, taking place chiefly by way of the olfactory cells, and that within 2 minutes pigment has passed into lymphatics and blood vessels, into perineural

sheaths and between nerve fibers and thence to the subarachnoid space and pia mater over the olfactory bulb. These latter findings, together with similar tests with bacteria and viruses, are described in the present paper.

Passage of Prussian Blue through the Olfactory Mucosa

10 per cent solutions of iron ammonium citrate and potassium ferrocyanide are prepared before each experiment. Equal amounts are mixed and the resulting particulate suspension is allowed to fall in small droplets on the outside of the nostrils whence it is breathed into the nose. In this manner 0.02 cc. is administered to each mouse—0.01 cc. in each nostril—without trauma or increased pressure within the nose. The mice are decapitated at intervals after inoculation. The head, without lower jaw and tongue, is placed in 10 per cent formalin containing 5 per cent HCl which acts as a decalcifying agent and also precipitates the pigment. After 5 days the heads are removed and divided sagittally along the midline. The halves are embedded in paraffin. Sections are prepared, lightly stained with Mayer's carmine and mounted.

Three experiments have been carried out. In the first, 20 Swiss mice were treated and 2 were decapitated at 2, 5, 15 and 30 minutes, 1, 2, 4, 6 and 8 hours. In a second test 4 mice of a strain susceptible both to bacterial and virus infections and 4 mice of a strain resistant to both types of infection were used (6), 2 of each group being sacrificed at 2 and 15 minutes. In the third test 12 mice of a bacteria-susceptible-virus-resistant strain, and 12 of a bacteria-resistant-virus-susceptible strain were used (6), 6 of each group being killed at 2 and 15 minutes after inoculation.

In the first experiment with 20 Swiss mice, the 2 killed within 2 minutes of inoculation showed widespread distribution of the dye. The picture seen at 1, 2 and 3 hours after inoculation (1, 2) is one only of end-results.

2 Minutes.—The pigment lies thickly spread over the surface of both olfactory and respiratory (ciliated) epithelium and is mixed with mucus, the latter being in smaller amounts than are found later.

The granules are passing through the olfactory mucosa. On first glance they appear to lie in narrow channels (Fig. 1) between the supporting cells, but on closer examination these streams of pigment are found to be clustered around the fibrils of the olfactory neurones which run to the free surface of the mucosa. Furthermore the granules fill the cytoplasm, but not the nucleus, of the neurone (Figs. 2, 3, 4). In some cases, moreover, the whole cytoplasm, but never the nucleus,

besides containing granules, is stained a diffuse blue. The streams of pigment can never be traced into the zone of mucosa next to the basement membrane, *i.e.*, below the layer of neurones, where they should appear if passing in intercellular channels. Thus while scattered granules may pass in between the cells, the greater part appear to travel through the olfactory cells themselves.

Over the respiratory mucosa the granules are often thickly tangled in the cilia. Some ciliated cells are stained diffusely blue and the nuclei are filled with granules. Such cells appear to be dead or damaged. Granules also appear in the cytoplasm of healthy ciliated cells or between the bases of such cells, showing that absorption is taking place through and between the respiratory cells. No absorption occurs through squamous epithelium.

The crypts of the serous glands contain pigment, and fine granules can occasionally be seen in the cytoplasm of the secreting cells.

In the submucosa the granules are scattered widespread in tissue interstices or, infrequently, inside cells. For the most part they line, or even fill a network of ramifying thin walled channels with all the characteristics of lymphatics (Fig. 5). These channels are especially full below an area of mucosa where absorption is most active. They empty into larger channels or penetrate the cartilaginous septum. Capillaries and venules contain scattered granules, while sinusoids in the septum or turbinates often show many. Capillaries or small venules, accompanying branches of the olfactory or trigeminal nerve or running in separate foramina through the cribriform plate, contain scattered granules.

The perineural spaces of the small branches of the olfactory nerve, supplying areas of mucosa where absorption is most active, are filled with granules of Prussian blue and the pigment lies between the nerve fibres in the middle of the nerve (Fig. 6). This marked concentration of pigment is confined to these small branches. The terminal twigs supplying the mucosa show scattered granules along their length and the main branches which pierce the cribriform plate, while showing granules in the perineural sheath and between the fibres, contain much less than the small branches. A few granules adhere to the nerve as it crosses the subarachnoid space to the olfactory bulb but are never seen within the brain itself save, very seldom, inside the small vessels. Branches of the trigeminal nerve show granules between the fibrils, in the perineural space or outside the perineural sheath. The granules are far fewer than along the olfactory branches.

The most surprising feature is in the subarachnoid space and the pia-arachnoid membrane of the olfactory bulb. Even at this early time the arachnoid network in the anterior angle of the skull contains granules (Fig. 7) as does the pia-arachnoid membrane (Figs. 8 and 9). Sinuses beneath the dura, especially the cavernous, contain much pigment.

5 Minutes.—Absorption is still active through both the olfactory and the respiratory mucosae but is less than at 2 minutes. The amount of mucus on the surface has increased. In the submucosa the lymphatics are often filled and the blood vessels contain more pigment than before. Pigment has decreased in the

small branches of the olfactory nerve but in the main branches the granules are as frequent as at 2 minutes, though the majority now lie in the perineural sheath rather than between the fibres. Some pigment is still to be seen in small branches of the trigeminal nerve. In the subarachnoid space the granules are as plentiful as at 2 minutes but fewer are seen in the pia-arachnoid membrane.

15 Minutes.—Absorption through the olfactory mucosa is considerably less. Through the respiratory mucosa it has almost ceased. Everywhere in the submucosa, save in the sinusoids of the turbinates, the pigment has greatly diminished. A few branches of the olfactory nerve contain as much pigment as at 2 minutes, but for the most part the pigment occurs only in scattered clumps in the perineural sheath. Granules are scattered in the subarachnoid space as far posteriorly as the pituitary fossa, and over the dorsum of the cerebrum. A few clumps are still present in the pia-arachnoid membrane.

From now on there is a steady decrease of pigment everywhere in the tissues although some absorption is still taking place.

8 Hours.—This is the longest time interval prior to examination. Very slight absorption is still taking place through and between the olfactory neurones. Masses of pigment, entangled in mucus, lie over the mucosa. In the submucosa a very few granules appear in the interstices or in lymphatics below areas of mucosa where absorption is still occurring. A few branches of the olfactory nerve show small clumps of pigment in the perineural sheath and similar clumps appear in the subarachnoid space over the olfactory area and anterior cerebrum. Scattered granules are still present in the pia-arachnoid over the tip of the olfactory bulb.

The Swiss mice used for the above test are very susceptible to certain bacteria and viruses given intranasally. Breeds of mice varying in their innate resistance to these bacteria and viruses were available in the laboratory (6) and were tested by the same technique. Previous results (4) had suggested that the innate difference in resistance in these breeds lay somewhere in the tissues rather than at the body surface.

Four BRVR (bacteria- and virus-resistant) and 4 BSVS (bacteria- and virus-susceptible) were used. They were inoculated intranasally with 0.02 cc. of 10 per cent Prussian blue by the technique described above. 2 of each group were decapitated at 2 minutes and 2 at 15 minutes after inoculation. Sections were prepared exactly as in the Swiss mice.

2 Minutes.—The picture in the BSVS mice resembles that described at 2 minutes in the Swiss. The BRVR mice show certain quantitative differences in that there are fewer granules both within the perineural sheath of the small branches of the olfactory nerve and in the subarachnoid space and pia-arachnoid membrane.

15 Minutes.—Again the picture in the BSVS mice resembles that seen at 15

minutes in the Swiss. The BRVR mice, however, show considerably more in the small branches of the olfactory nerve and in the intracranial cavity. Indeed the picture resembles that seen in the Swiss and BSVS animals at 2 minutes.

It appears, therefore, that both susceptible breeds respond alike. In the resistant animals, however, passage into the tissues and vessels occurs as rapidly as in the susceptibles, but passage into the nerves and thence to the subarachnoid space is slightly delayed. This observation was made with only few mice and its significance is doubtful.

When the experiment was repeated with BRVS and BSVR mice (12 in each group and 6 of each sacrificed at 2 minutes and 6 at 15 minutes) no difference could be found in either group from the picture described in the Swiss and BSVS mice.

Olitsky and Cox (2) found absorption of pigment less evident in tannic acid-treated mice than in normal ones studied 1 and 3 hours after inoculation of Prussian blue. We repeated the experiments and examined mice within a few minutes after instilling the dye. The results have been reported briefly elsewhere (7).

Eight treatments of 0.03 cc. of 0.8 per cent tannic acid in 1 per cent glycerine were given during 3 days to 9 Swiss mice. The mice were given intranasally 0.02 cc. of the Prussian blue mixture 4 hours after the last tannic acid treatment. 5 mice were sacrificed 2 minutes and 4 mice 15 minutes later. Microscopical preparations were made in the way described above. Most were stained with Mayer's carmine; a few with hematoxylin and eosin.

Examination revealed two changes from the picture seen in untreated mice. First, although tannic acid-treated mice rarely show any exudate from the nose during life, actually, the nasal cavities over the olfactory mucosa and around the turbinates were filled with a heavy exudate of polymorphonuclear cells lying in thick mucus. The mucosa itself and submucosa were infiltrated with fluid and cells; the capillaries in the submucosa were dilated and contained many leucocytes.¹ The respiratory mucosa showed little change.

The absorption of pigment at 2 and 15 minutes was only slightly less than in untreated mice. Granules were as plentiful in the tissue spaces and in the vessels, but there was quantitatively less pigment

¹ The inflammation occurs when 1 per cent tannic acid in normal saline is used and is due therefore to the tannic acid rather than the glycerine.

along the branches of the olfactory nerve, in the subarachnoid space and in the pia-arachnoid than is seen in untreated mice at these times.

The second striking departure was found in the olfactory mucosa. In the tannic acid-treated mice the selective passage of pigment into the olfactory neurones was not seen. In 6 of the 9 mice these cells contained no granules and in 3 only a few olfactory neurones contained granules. The 6 mice showing no pigment in these neurones were those with the most intense inflammation. In the other 3 the inflammation was less. A causal relationship between the inflammation and the absence of passage of pigment into the neurones has not been shown; it seems more probable that both departures from the normal are parallel responses to the tannic acid.

The above studies show that pigment instilled intranasally in the mouse is absorbed very rapidly. Absorption occurs chiefly through the olfactory mucosa where the pigment selects the olfactory neurones. Within 2 minutes pigment is present inside vessels and within the perineural sheath of the olfactory nerve along which it has reached the subarachnoid space and pia mater. Subsequently it slowly disappears. Absorption of pigment is not prevented by preliminary tannic acid treatment. Such treatment does decrease the amount of pigment reaching the perineural space, the subarachnoid space and the pia mater, but its principal actions are to cause an acute inflammation of the olfactory mucosa and an almost complete interruption of the passage of pigment into the olfactory neurones.

Passage of Bacteria through the Olfactory Mucosa

It has been shown (4) that pneumococci instilled intranasally in mice appear very rapidly in the peripheral blood. The results suggested that this invasion occurred both through the alveoli of the lung and also through the olfactory mucosa.

It was decided to investigate further the distribution of pneumococci in the body in the early periods after intranasal instillation. If they behave like pigment, and if invasion occurs through the olfactory mucosa, one should obtain early positive cultures from the olfactory area of the brain even in the absence of positive cultures in blood and other organs.

Mice were given 0.02 cc. of a 16 hour broth culture of intranasally virulent Type III pneumococci by the usual technique. At given times after inoculation they were sacrificed and various tissues cultured for pneumococci. In removing the brain, from which only the olfactory area was cultured, great care was taken to avoid opening the nasal cavity from above. The results of cultures from blood, spleen and olfactory area, in 4 tests using 31 mice sacrificed at periods up to 30 minutes, are shown in Table I. During this time 6 mice showed positive blood or spleen cultures and 23 positive olfactory area cultures.

The experiment was repeated using *Salmonella enteritidis*. 2 tests with 51 mice were carried out. Blood and olfactory area were cultured for bacteria at intervals up to 30 minutes. From Table I it will

TABLE I

Organism	Organ	Time in minutes													
		1	2	3	4	5	6	8	10	12	15	20	22½	25	30
Pneumo- cocci	Blood and/or spleen	0/4*	2/4			0/4		0/4	1/5		0/2	2/3	0/1		1/4
	Olfactory area of brain	3/4	2/4			3/4		3/4	5/5		0/2	3/3	1/1		3/4
<i>B. enteritidis</i>	Blood	0/3	1/3	1/3	0/3	0/3	0/3	0/3	1/6	0/3	1/6	2/6		0/3	0/6
	Olfactory area of brain	1/3	2/3	1/3	1/3	1/3	1/3	0/3	4/6	2/3	2/6	3/6		2/3	4/6

* 0/4 = no positive cultures out of 4 taken at this time interval.

be seen that 6 mice gave positive blood cultures and 24 positive olfactory area cultures.

Both a respiratory and an intestinal pathogen, therefore, can reach the brain shortly after contact with the olfactory mucosa. The 4:1 preponderance of positive olfactory area cultures over those obtained from the blood or spleen seems to indicate that the former are not due to bacteremia.

Studies were made² to determine whether tannic acid in dilutions effective against virus diseases (2) could protect mice against intranasal instillation of pneumococci. The results reported elsewhere (8) show that such preliminary treatment gave no protection against pneumococci. During these studies it was found that the pneu-

² These studies were made with Dr. H. R. Cox as collaborator.

mococci reached the blood as rapidly in the tannic acid-treated mice as they did in the untreated animals. Subsequently we have found that the pneumococci reach the olfactory area as rapidly in treated mice as they do in untreated. The tannic acid treatment therefore does not influence the passage of pneumococci through the olfactory mucosa.

In order to study the actual passage of pneumococci through the mucosa 4 mice were each given by the usual technique 0.03 cc. of a 12 hour broth culture of Type III pneumococcus, centrifuged down and resuspended in one-third the original volume. 2 minutes later all mice were decapitated. Fixation and decalcification was done in 10 per cent Zenker's acetate fluid. Sections were prepared as above but stained with Giemsa's stain.

Microscopically pneumococci are seen lying on the surface of the mucosa between fibrils or cilia. In the olfactory region some lie between cells near the surface but the majority lie between supporting cells close to the basement membrane. All invasion seems to be between the cells. In the submucosa pneumococci are seen in the tissue spaces, some inside phagocytes, in the lymphatics and, to a lesser degree, in the capillaries. They also appear in the perineural space of the olfactory but not the trigeminal nerve and are frequent in the subarachnoid space and within cells of the pia mater. Invasion between cells of the respiratory mucosa occurs but is considerably less.

Distribution tests and direct microscopical examination show therefore that pneumococci and *S. enteritidis* enter the body through the olfactory mucosa very rapidly and, like the pigment, immediately reach the intracranial cavity. Passage through the mucosa is accomplished between the cells and not by way of the olfactory neurones. This passage is in no way hindered by preliminary tannic acid treatment.

Passage of Viruses through the Olfactory Mucosa

Following the results with Prussian blue and bacteria the behavior of viruses inoculated by the same technique has been studied. Tests have been carried out with four viruses (Table II).

Mice are given 0.03 cc. of a suspension of the test virus intranasally by the usual technique. Virus suspensions are prepared in hormone broth usually at a dilution of 1:5 but in 2 cases at 1:10 and 1:100. At given times after inoculation the mice are killed and the tissues tested for virus. Usually the olfactory area of the brain and the spleen are tested. In 2 cases the spleen was omitted and in

TABLE II

TABLE II

Virus used and dilution	Organ tested	Minutes												Hours			Days			
		1	2	3	4	5	6	7	8	10	30	1	3	6	1	2	3	1	2	3
St. Louis encephalitis 1/10	Olfactory area		0, 0							0, 0	0, 0	0, 0	0, 0			5, 6	5, 8			
St. Louis encephalitis 1/100	"		0, 0							0, 0	0, 0	0, 0	0, 0			0, 0	4, 5			
St. Louis encephalitis 1/5	Spleen		0, 0	0, 0		0, 0	0, 0	0, 0								6, 11				
	Olfactory area		†, 0	0, 0		0, 0	0, 0	0, 0								7, 0				
Louping ill 1/5	Spleen		0, 0		0, 0		0, 0		0, 0	0, †		0, 0	0, 0			0, 0			0, 0	0, 0
	Olfactory area		0, 0		0, 0		0, 0		0, 0	0, 0		0, 0	†, 0			8, 8			8, 9	
Louping ill 1/5	Spleen		0, 0		0, 0		0, 0		0, 0	0, 0		0, 0	†, 0			0, 0			0, †	
	Olfactory area		0, 0		0, 0		0, 0		0, 0	0, 0		0, 0	0, 0			10, 13			8, †	
Eastern equine encephalomyelitis* 1/5	Spleen	0, 0	0, 0		0, 0		0, 0		0, 0	0, 0						0, 0			0, 0	
	Olfactory area	0, 0	3, 3		3, 4		0, 0		0, 0	0, 0						0, 0			3, 3	
Eastern equine encephalomyelitis 1/5	Spleen + heart's blood	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0									0, 0			0, 0	
	Olfactory area	0, 0	2, 2	0, 0	2, 4	3, 4	0, 0									0, 0			2, 2	
Rabies 1/5	Spleen + heart's blood			0, 0	0, 0		0, 0		0, 0	0, 0										
	Olfactory area		0, 0	0, 0	0, 0		0, 0		0, 0	0, 0										

2 mice injected intracerebrally with each test material. Blanks indicate that no test was made. 0 = mouse survived well. 3, 4, etc., = mouse died from the specific infection on the 3rd, 4th, etc., day. † = mouse died from trauma during inoculation.

* We are indebted to Dr. Olitsky and Dr. Cox for this virus. Prior to the early positive results which we found with Eastern equine encephalomyelitis virus, Dr. Cox had noted the same phenomenon occasionally (unpublished work).

3, half of the fluid used for dilution consisted of heart's blood from the same mouse, used to increase the possibility of finding small amounts of virus in the blood. The tissues are ground and suspended in hormone broth 1:10. 2 mice are inoculated with 0.03 cc. of each test suspension.

It will be seen that the viruses fall into two groups. St. Louis encephalitis, rabies and louping ill viruses³ do not behave like pigment or bacteria but appear to be held up at the surface. The olfactory area of the brain is never positive until 24 hours after inoculation. Equine encephalomyelitis, however, resembles pigment and bacteria in reaching the intracranial cavity very rapidly. The fact that this virus is demonstrable only at 2 and 5 minutes after inoculation and then disappears is a further resemblance to the behavior of the pigment which reaches its maximum at 2 to 5 minutes. The virus reappears in the brain at 24 hours.

DISCUSSION

The above experiments suggest, *inter alia*, that the intranasal technique is more artificial than is usually supposed. By means of it substances are brought into widespread contact with the highly permeable surfaces in nose and lung (4) even when only 0.005 cc. is given. Such an exposure of mucosa does not occur normally in nature but represents, nevertheless, in exaggerated manner the slight mucosal contaminations which must be so frequent. Probably these normal contaminations are followed by rapid penetration of the mucosa, but their slowness and the resistance of the tissues limits the number of cases in which disease follows. That resistant hosts can deal with extensive preliminary invasion by bacteria is shown by the fact that mice resistant to Type III pneumococci given intranasally show as frequent early positive blood cultures as do susceptibles which die of the infection (4). During the present study it has been found that resistant mice show as frequent positive cultures from the olfactory area of the brain after intranasal instillation of pneumococci as do the susceptibles (Table III).

Pigment, bacteria and at least one virus given intranasally reach the tissues, the circulation and the brain very rapidly. In the last

³ According to a test carried out by Dr. A. B. Sabin, vesicular stomatitis virus (New Jersey strain) belongs to this group (personal communication).

region the maximum concentration is reached in from 2 to 5 minutes. Pigment enters largely through the olfactory neurones but the bacteria appear to pass between the mucosal cells. The fact that tannic acid, which prevents the passage of pigment into the olfactory neurones, offers protection against certain viruses, suggests that the latter may enter these olfactory sensory cells as does the pigment.

The experimental results suggest an explanation of the fate of viruses instilled into the nose. If neurotropic viruses do enter the olfactory neurones like the pigment, they would tend rather to be held here than to pass freely to the tissues and the brain. Experimentally they have not been found in the brain immediately. The pantropic viruses,

TABLE III

	Organ	Time in minutes						
		1	2	5	7½	10	20	30
Resistant mice	Spleen	0	+	0	0	0	0	0
	Blood	0	+	0	0	0	0	0
	Olfactory lobe	+	+	+	0	+	+	+
Susceptible mice	Spleen	0	+	0	0	0	+	+
	Blood	0	+	0	0	0	+	+
	Olfactory lobe	+	+	+	+	+	+	+

0 indicates negative culture.

+ indicates positive culture.

however, should be able to pass freely out of the olfactory cells and experimentally one such virus has been shown to reach the brain immediately. This virus, equine encephalomyelitis, then disappears, probably being dispersed as is the pigment, and it reappears later. This disappearance may mean that the concentration is lowered below that needed to infect another mouse, but enough virus may remain to multiply and become redemonstrable later on. On the other hand the early invasion may be unimportant and may be effectively dealt with by the tissues, while the true infection progresses only slowly from the periphery. Slow progression seems to occur with the neurotropic viruses. It is still doubtful how this spread occurs, but it may be said that the evidence presented is contrary to Findlay's (9) and Hurst's (10) view of a slow progression in the perineural space.

SUMMARY

1. Prussian blue particles pass rapidly from the surface of the olfactory mucosa and within 2 minutes are found in the tissue spaces, in blood and lymph vessels, in the perineural spaces of the olfactory nerve fibers and in the subarachnoid space and pia-arachnoid membrane.

2. There is great affinity of pigment particles for the olfactory sensory cells.

3. Preliminary treatment of the olfactory mucosa with tannic acid does not alter the speed with which this absorption occurs. It does, however, cause an inflammation of the mucosa and appears to prevent the pigment from entering the olfactory sensory cells.

4. Both pneumococci and *S. enteritidis* pass through the olfactory mucosa and reach the tissue spaces, the vessels and the subarachnoid space with the same rapidity as the pigment. This can be demonstrated both microscopically and by distribution tests. They invade by passage between the cells of the mucosa and there is no apparent affinity of the organisms for the olfactory sensory cells.

5. Tannic acid treatment of the olfactory mucosa in no way alters this invasion of organisms through the mucosa.

6. The pantropic virus, equine encephalomyelitis, was detected in the forebrain as promptly as were pigment and bacteria; neurotropic viruses, however,—those of St. Louis encephalitis, rabies and louping ill,—were not demonstrated in less than 24 hours.

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EXPLANATION OF PLATES

PLATE 8

FIG. 1. 2 minutes. Mucous membrane. Pigment massed on the free surface of the olfactory mucosa and appearing to pass in streams between the superficial cells. In the submucosa pigment can be seen in the tissue interspaces. $\times 1000$. Mayer's carmine.

FIGS. 2, 3 and 4. 2 minutes. Mucous membrane. In Figs. 3 and 4 the pigment is massed on the free surface of the olfactory mucosa. In all olfactory sensory neurones are shown with numerous pigment granules in their cytoplasm. Granules can be seen following along the sensory fibrils of 3 cells which run to the free surface. In Figs. 2 and 4 granules are seen in the submucosa. $\times 1000$. Mayer's carmine.

PLATE 9

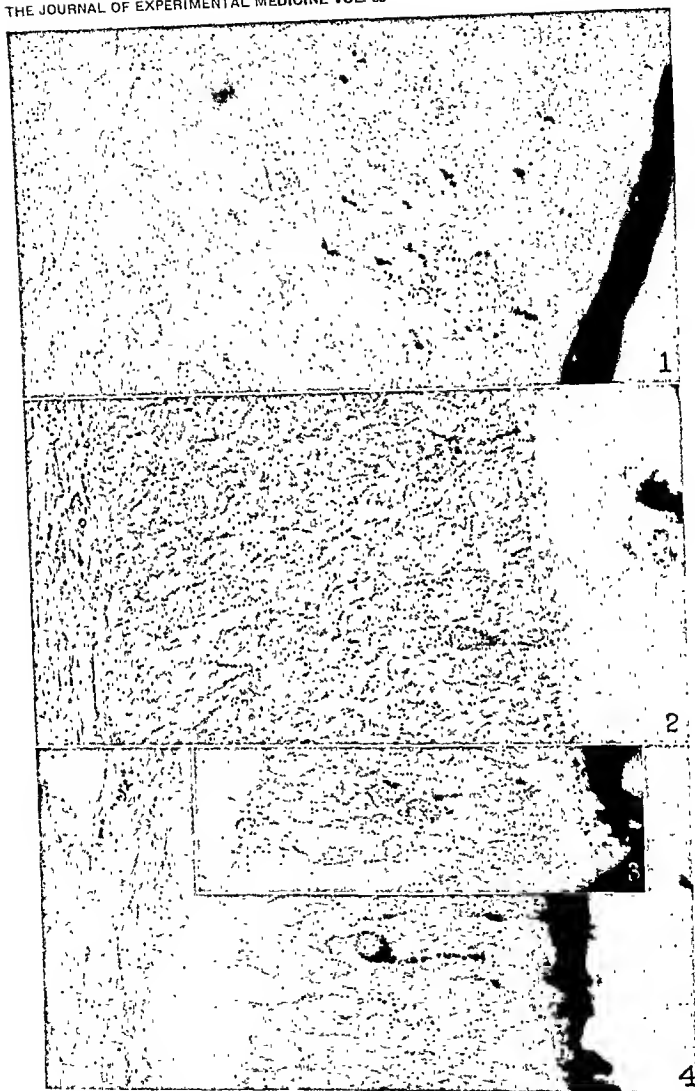
FIG. 5. 2 minutes. Submucosa. Ramifying lymphatic channels filled with granules. Some granules in larger blood vessels. To the right is a large branch of the olfactory nerve with pigment in the perineural space and between the fibres. $\times 400$. Mayer's carmine.

FIG. 6. 2 minutes. Submucosa. A bifurcating branch of the olfactory nerve. The perineural space and the interfibril spaces are packed with Prussian blue granules. (This nerve appeared as a blue band to the low power of the microscope.) Above is a venule with pigment granules lining the endothelium. $\times 1000$. Mayer's carmine.

PLATE 10

FIG. 7. 2 minutes. Subarachnoid space. Pigment granules, scattered or in masses, occupying the subarachnoid network at the anterior angle of the skull. $\times 1000$. Mayer's carmine.

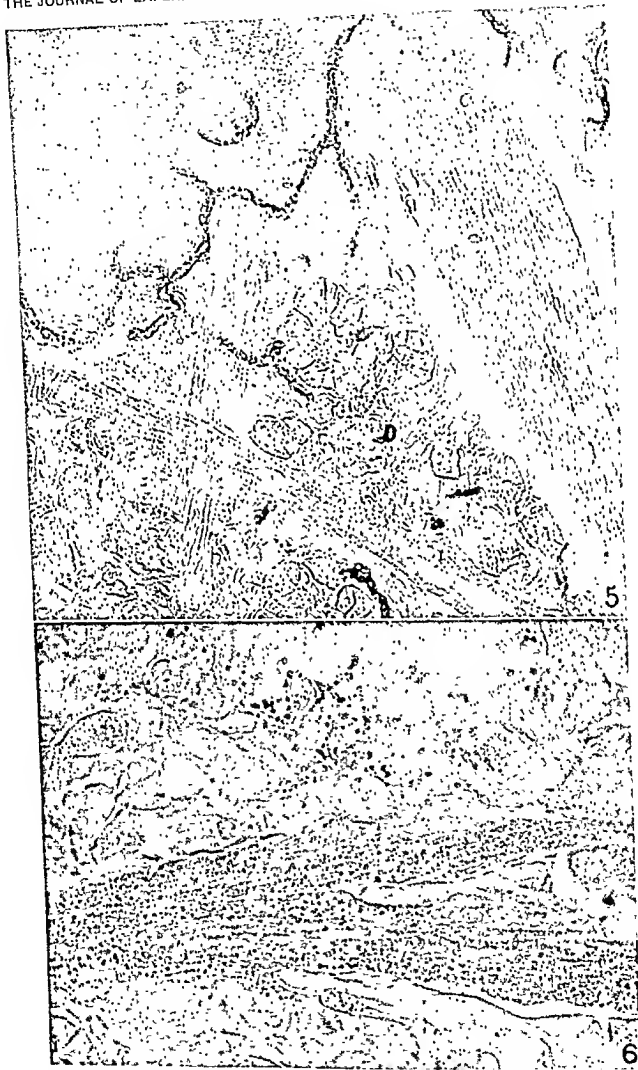
FIGS. 8 and 9. 2 minutes. Pia mater. Pigment granules in and between the cells of the pia over the olfactory bulb. None in the substance of the brain which lies below in both figures. $\times 1000$. Mayer's carmine.

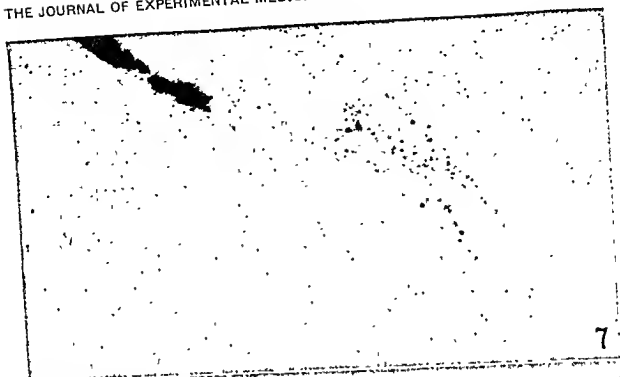


Photographed by Louis Schmidt

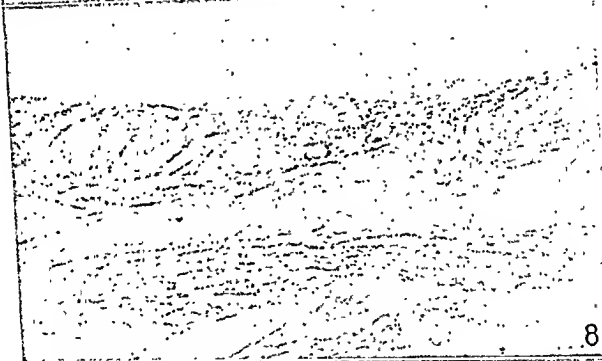
(Rake: Invasion of body through olfactory mucosa)



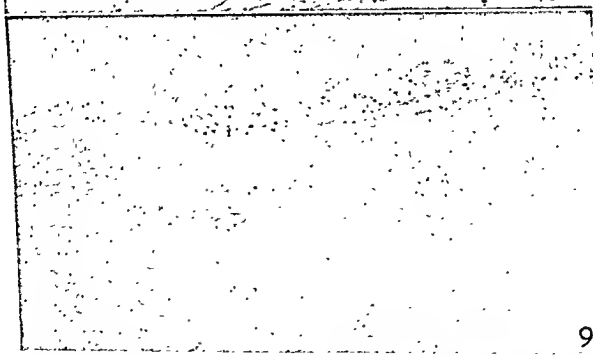




7



8



9

Photographed by Louis Schmidt

(Note: Invasion of body through alveolar spaces)

STUDIES ON MENINGOCOCCUS INFECTION

X. A FURTHER NOTE ON THE PRESENCE OF MENINGOCOCCUS PRECIPITINOGENS IN THE CEREBROSPINAL FLUID

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In earlier communications (1, 2) there was described a rapid method for diagnosing meningococcal meningitis and ascertaining the serological type of the organism by means of precipitin tests carried out on the cerebrospinal fluid with monovalent antisera. It was pointed out that a certain number of negative results occurred when the spinal fluid tested was drawn after the commencement of intrathecal serum therapy, and, furthermore, that in some instances negative results were due apparently to the fact that the test was not sufficiently sensitive to demonstrate the very small amounts of specific precipitinogen which might be present in some fluids. At that time only rabbit monovalent antimeningococcus sera were available. It was thought that the percentage of negative results on fluids drawn before commencement of therapy might be reduced when sera of higher precipitin titer became available.

Since the publication of these papers there has become available both Type I and Type II monovalent antimeningococcus serum, prepared in horses, which has a precipitin titer considerably higher than our best monovalent rabbit serum. These sera have been used in the tests carried out on spinal fluids since that date. Moreover, the technique of the precipitin test has also been modified so as to give more delicate readings.

Material and Technique

Spinal fluid was obtained from cases of known meningococcal meningitis and from other cases of meningitic infection. The precipitin test was carried out with

equal parts of undiluted serum and spinal fluid cleared by centrifugation. The tubes were kept at 37°C. for 2 hours and the ring method was used for reading the results. Only Type I-III and Type II sera were used. Type IV serum was omitted since no strains of this type are being seen at this time and only an old stock strain was available for serum production (2); and Type III serum was not used since additional work has abundantly confirmed the opinion, given elsewhere (3), that there is no significant difference between Types I and III, so called, as they occur today.

0.1 cc. of each of the two monovalent sera is placed in a small tube of 4 mm. inner diameter and 5 cm. length. 0.1 cc. of the spinal fluid to be tested is carefully layered on top of each serum so that a clear line of junction is formed between the two fluids. Any precipitate which forms can be seen as a distinct white ring at the point of junction, and, given the proper lighting conditions, which are described below, can be seen with ease. A reading is made immediately and the tubes are then placed in the 37°C. water bath. Further readings, without mixing of the fluids, are made at the end of 1 and 2 hours. In order to see slight amounts of precipitate it is essential to use suitable lighting conditions. The best arrangement has been found to be the following. A microscope lamp is used which casts a strong bluish white beam of light. This is placed slightly above and to the left of the line of horizontal vision and is so arranged that the beam of light is cast obliquely down and to the right across the line of vision. The tubes are held in this oblique beam of light on a level with the eye and are examined against a dull black background. Other means of lighting are as efficacious but the one herein described has proved satisfactory.

RESULTS

Following the introduction of the higher titer Type I-III and Type II horse sera and the modification of technique, all spinal fluids from cases of meningitis received in the laboratory have been tested for their precipitin content with these sera. While the results have shown a high percentage of positive tests, there have been a certain number of negative reactions in fluids which would be expected to be positive (Table I). Moreover there has been a lack of agreement between the results obtained in our hands and in certain laboratories attached to the hospitals where the fluids originated, although the same sera were available in all places and the technique employed is believed to have been the same. Since the lack of agreement always lay in our obtaining precipitin reactions on fluids which other laboratories had found negative, it seemed probable that some change must be occurring during the time between the two tests and that this change might be lysis of the organisms, with or without continued growth during transit of the culture.

Recently one of us has been in a position to obtain fluids from a large outbreak of meningococcal meningitis due to Type I-III organisms.¹ These fluids have been studied by the technique given above, using the antimeningococcal horse sera. The results on 70 spinal fluids from proven cases of meningococcal meningitis show in brief that: 36 fluids reacted at once with Type I-III serum; 29 did not react at once; 4 reacted when first tested after 24 hours incubation; 1 reacted when first tested after 48 hours at room temperature. Of the 29 fluids which did not react at once: 5 reacted after 24 hours incubation; 13 reacted after 48 hours at room temperature (not tested earlier); 4 reacted after 48 hours but not after 24 hours at room temperature; 7 remained negative. Of the 36 fluids reacting at once, 22

TABLE I

Type	No. of fluids positive in homologous serum	No. of fluids negative in both sera
I-III	32	3
II	10	0
II*	2	1
VII	1	2
Other forms of meningitis	0	14

No heterologous precipitin reactions occurred. The positive reaction given in fluid from atypical Type II* or Type VII cases was in Type II serum and was very slight. Such a reaction is to be expected (4).

gave a definite ring precipitate within 10 minutes of the two fluids coming into contact, thus allowing immediate diagnosis and typing to be made. In the 14 other fluids the precipitate appeared by the end of 1 hour in the 37°C. water bath.

It seems from this series that: 33.8 per cent of fluids can be typed at once; 21.5 per cent within 1 hour; 7.7 per cent within 1 day; 20.0 per cent between 1 hour and 2 days; 6.2 per cent within 2 days; and that 10.8 per cent of fluids never give positive reactions. This corresponds with the figures given in Table I where 3 out of 35 fluids from Type I meningococcal meningitis, or 8.6 per cent failed to give positive reactions.

¹ Our thanks are due to Dr. Huntingdon Williams and the other members of the Baltimore City Department of Health whose hospitality and help made this work possible.

It was further noted that in those cases in which the fluid gave an immediate reaction, the course of the disease was severe and the prognosis grave, while in those cases in which the fluid failed to react or became positive only after 1 hour to 2 days, 95 per cent recovered. This will form the subject of a further report (5).

Specimens of concentrated Type I and Type II monovalent sera have been used in the precipitin test with the spinal fluid in some instances. It was thought that such sera might prove even more sensitive in the demonstration of minute amounts of specific precipitogens. The results are given in Table II. It will be seen that

TABLE II

Name or number	Type I serum	Type I serum concentrated	Type II serum	Type II serum concentrated	Agglutination	Notes
C.	+	++	0	±	I-III	Before intrathecal serum
C.	0	0	0	0	I-III	After intrathecal serum
W.	0	0	0	0	I-III	After intrathecal serum
505	++	+++	±	±	I-III	
506	++	+++	0	±	I-III	
F.	0	±	±	+	II	
504	0	±	+	++	II	
498	0	±	±	+	II*	
500	0	±	±	+	II*	
501	0	0	0	0	VII	
I	0	±	0	±	Pneumococcus 10	

II* = atypical Type II strain.

the homologous precipitin reactions evoked are stronger than those due to the unconcentrated sera. However, this advantage is more than offset by the heterologous reactions which occur. It appears that the methods of concentration thus far adopted have resulted in a concentration not only of the specific antibodies in the serum but also of the non-specific C and P antibodies. This gives rise to cross reactions between the two meningococcus types and even in one case to a reaction with a fluid from a case of pneumococcus meningitis. This latter result was to be expected on the basis of previous work (6).

SUMMARY

Precipitin tests have been carried out on spinal fluid from cases of meningococcal and other forms of meningitis, with monovalent anti-meningococcus horse serum of high titer. Using such a test it has been possible within 2 hours to diagnose and type cases of Type I and Type II meningococcal meningitis. In a certain number of cases fluids which were negative when first drawn became positive after standing for 1 or 2 days at 37°C. or room temperature. In 9.5 per cent of all Type I cases the fluids did not become positive. Fluids from cases due to atypical meningococci may react with the type serum of the group to which they belong (*i.e.*, Types II* and VII, which belong to group II, with Type II serum) but do not in every case. Fluids from forms of meningitis other than meningococcal give no reaction. The use of concentrated sera is not advantageous at present, owing to the heterologous reactions which occur.

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CHEMICAL INVESTIGATIONS ON THE ACTIVE PRINCIPLES OF THE PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES

I. PURIFICATION BY DIALYSIS, AND ATTEMPTS AT FRACTIONAL PRECIPITATION*

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(Received for publication, November 14, 1936)

Chemical investigations on the active principles of the phenomenon of local skin reactivity to bacterial filtrates have only recently been undertaken.

Stable meningococcus and *Bacillus typhosus* phenomenon-producing principles have been obtained by precipitation of culture filtrates with ammonium sulfate, dialysis, and drying *in vacuo* (Shwartzman (1)). The phenomenon-producing potency of *Streptococcus hemolyticus* cultures and culture filtrates has been preserved by precipitation with acid alcohol (Shwartzman (2)). Bordet (3) concentrated the active principles from broth cultures of *B. coli* by precipitating them with acetic acid and redissolving the precipitate in a small volume of water. Ecker and Welch (4) prepared potent materials by decomposition of a lead precipitate of *B. paratyphosus* B filtrates by means of ammonium sulfate and dialysis (method of Ecker and Rimington (5)). Gentile (6) reported briefly upon the activity of alkaline extracts of *B. coli*. Linton, Singh, and Seal (7) found the highest skin-preparatory potency and specific antigenicity in fraction A, obtained by the dissolution of cholera vibrio at 50°C. in alcohol made 0.025 N with HCl. The material had a specific rotation of about -12.0° and a nitrogen distribution which was characterized by the possession of about twice the amount of amide nitrogen and one-half the amount of humin nitrogen as in the whole protein. Fraction B, obtained by boiling vibrios in alcohol and the residue fraction, consisting of the material which remained after the second acid-soluble fraction had been removed, proved inactive.

According to Joukow-Werejnikow and Lipatova (8), the nucleoprotein

* This investigation has been aided by a grant from Eli Lilly and Company, Indianapolis.

fractions (method of Lustig and Galeotti) and water extracts of *B. pestis* containing the capsular material gave negative results whilst the "agar washings" filtrates of encapsulated *B. pestis* were endowed with high potency. Olitski and Leibowitz (9) attempted to produce the phenomenon with fractions P₁, P₂, and C of Furth and Landsteiner (10). The P₁ (crude) and C fractions apparently possessed very small amounts of reacting factors, whilst P₁ purified and P₂ fractions contained most of the active principles.

Andervont and Shear (11) purified *B. coli* filtrates by means of basic calcium phosphate precipitation (method of Felton, Kaufman, and Stahl (12)). Their purest preparation gave a positive Molisch test and negative biuret test, and exhibited activity at 0.0005 mg. per unit.

By means of precipitation with various reagents, Apitz (13) obtained several fractions which were endowed with skin-preparatory potency. He made no studies on the reacting (intravenous) factors. As a starting point for chemical investigations, Apitz's procedure of fractional precipitation was adopted. In the present communication, however, the reacting potency of the various fractions was evaluated quantitatively; in many cases the skin-preparatory potency was also noted.

It was soon discovered that during these operations of concentration, most of the activity originally present in a bacterial filtrate was lost. Even in preparations which retained their potency, analysis (activity per milligram of nitrogen and of dry weight) showed that only an apparent concentration had been effected. Other methods of purification were therefore investigated. The simple operation of dialysis immediately proved very valuable for this purpose.

The work embodied in this paper comprises a quantitative biological assay of the materials procured in (1) attempts at fractional precipitation by means of the methods used by Apitz; and (2) the purification of active filtrates by dialysis.

Methods

Inasmuch as the active principles of the phenomenon under discussion may be produced by certain bacterial contaminants, the maintenance of a strictly aseptic technique throughout the investigation was considered of paramount importance. Intermediate and final preparations were tested on a variety of aerobic and anaerobic media; similarly, solutions were always tested before and after dialysis. Contaminations were rarely obtained. The contaminated preparations were discarded.

Nitrogen analyses were conducted by the Parnas-Wagner modification of the Pregl micro Kjeldahl method. Total dry weights were determined by pipetting 1 cc. of the sample into a tared aluminum dish and heating to constant weight in an oven at 102–103°C. An overnight period proved sufficient and convenient for this purpose. Determinations of dry weight and nitrogen were always made in duplicates, which usually agreed within 3 per cent.

All of the filtrates, as well as various preparations derived from them, were tested quantitatively for reacting potency, using the methods previously described (14). Wherever indicated, the skin-preparatory potency was also determined, using 25 units of "agar washings" filtrate for the eliciting intravenous injection. In most of the experiments, when a given preparation was tested for potency, either skin-preparatory or reacting, a standardized quantity of the original batch of filtrate was used for the provocative or skin-preparatory injection, respectively.

When an active filtrate was titrated to the end-point, the titer computed was the mean of the dilutions at which positive and negative reactions were obtained. In calculating the percentage of recovery of activity, the maximum error of titration was used. For example, when injected intravenously, a certain filtrate (before dialysis) elicited positive reactions in prepared skin sites at a dilution 1:100 and negative reactions at a dilution 1:200. The reacting titer assigned to this filtrate, was, therefore, 150 ± 50 units per cc. The limits of the error thus were $\pm 50/150$ or ± 33 per cent. After dialysis, the filtrate gave positive results at dilutions 1:91 and negative results at dilutions 1:140. The titer computed was then $1:116 \pm 25$ reacting units per cc. In this instance, the limits of error were then $\pm 25/116$ or ± 22 per cent. During the dialysis of this filtrate, the volume had increased from 36 to 79 cc. The percentage of recovery, therefore, was:

$$\frac{(116)(79)(100)}{(150)(36)} = 170 \pm 57.$$

The error ± 57 , applied to the 170 per cent recovery, was obtained by taking 33 per cent of the latter, i.e., the maximum of the titration errors before and after dialysis. It is realized that this does not represent the maximum possible error.

EXPERIMENTAL

1. *Attempts at Fractional Precipitation of Active B. coli and B. typhosus Culture Filtrates.*—The various bacterial filtrates which were used are described below. The "agar washings" filtrates were prepared by methods previously described (15).

*T.2158. B. coli Synthetic Medium Culture Filtrate.*¹—*B. coli* was grown in syn-

¹ This filtrate was prepared by Eli Lilly and Company, Indianapolis.

thetic medium prepared as follows: 20 cc. glycerine, 5 gm. NaCl, 1 gm. K_2HPO_4 , 0.2 gm. $MgSO_4$, 1 gm. KH_2PO_4 , 0.1 gm. $CaCl_2$, and 20 gm. peptone, diluted to 1 liter. At the time of inoculation, sodium lactate and glucose were added to a concentration of 0.5 per cent and 0.2 per cent, respectively. The final pH was 5.8.

T.2157 and T.2163. B. typhosus T_L "Agar Washings" Filtrates.—Plain agar Kolle flasks, at pH 7.4, were inoculated with a 24 hour broth culture of *B. typhosus*, T_L strain, and incubated at 37°C. for 23 hours. 3 cc. of distilled water per Kolle flask were used for washing off the growth. The suspension was centrifuged and the supernatant solution filtered twice through Berkefeld V candles. The final pH was 8.4.

T.2145 and T.2154. B. typhosus T_L Plain Broth Culture Filtrates.—Plain veal infusion broth at pH 7.6 was inoculated with a 24 hour culture of *B. typhosus*, T_L strain, and incubated for 3 days at 37°C. The suspension was centrifuged and the supernatant solution filtered three times through Berkefeld V candles. The final pH of T.2145 was 7.5 and that of T.2154 was 7.0. Merthiolate was added to a final concentration of 1:15,000.

T.2146. B. typhosus T_L Plain Broth Culture Filtrate.—Identical to T.2145 and T.2154 described above, but four filtrations were necessary in order to obtain a sterile filtrate and merthiolate was added to a final concentration of 1:50,000. The final pH was 6.8.

The method of fractional precipitation was that described by Apitz (13). His terminology, furthermore, has been adopted.² The operations involved in this procedure were essentially as follows:

(a) An active filtrate was concentrated *in vacuo* at 37°C. to about one-tenth of its original volume and subsequently precipitated with 95 per cent ethyl alcohol. (b) The precipitate was dissolved in dilute sodium hydroxide, any insoluble material being designated as fraction N. (c) Acetic acid was added to the supernatant solution until precipitation was at a maximum; the latter was called fraction P. (d) The solution from (c) was then precipitated with about six volumes of 95 per cent ethyl alcohol. (e) When fractions N and P were completely removed by repetition of the above procedures, including several alcoholic precipitations from alkaline as well as from acid solutions, the final alcoholic precipitate was designated as fraction C.

To illustrate the technique employed in the present investigations, the following detailed description is given of a typical experiment with *B. typhosus* T_L T.2146 culture filtrate. 2720 cc. of filtrate T.2146 were concentrated *in vacuo* at a vapor temperature not exceeding 37°C. During this process, the fluid was allowed to stream steadily through a 3/8 inch bent glass tube into the bottom of a 5 liter Claisen flask. The use of a stream of air through a capillary tube was thus

² Similar designations by other authors may have reference to entirely different bacterial fractions.

avoided. In order to prevent foaming, 0.1 cc. of Du Pont antifoam No. L.F. was injected whenever necessary through the connecting rubber tubing by means of a syringe. In approximately 3 hours, the volume was reduced to 250 cc. The pH of the solution changed from 6.8 to 6.5 (bromothymol blue). 3 gm. of sodium acetate and 10 cc. of $N/1$ NaOH were added. The concentrate was poured immediately into 2 liters of 95 per cent alcohol, which were continuously stirred. A gummy precipitate formed which was allowed to remain overnight in the ice box in contact with the alcohol. The clear supernatant alcoholic solution was decanted. The precipitate was suspended in centrifuge bottles by thorough agitation with 230 cc. of water. 7 cc. of $N/1$ NaOH were added and after 15 minutes of stirring, the solution was centrifuged. The resulting precipitate, which was preserved under alcohol, was referred to as the first N fraction (N_1). When the clear supernatant solution was acidified, a fine precipitation occurred, which was apparently complete when 70 cc. of acid were added. The precipitate was removed by centrifugalization and designated as the first P fraction (P_1). The solution was poured into six volumes of 95 per cent ethyl alcohol and kept in the ice box overnight. The above procedure was then repeated, *i.e.*, dissolution in dilute NaOH (145 cc. water plus 10 cc. N NaOH), centrifugalization, precipitation with acetic acid (20 cc. of 20 per cent alcohol), centrifugalization, and finally precipitation with six volumes of alcohol. In this manner, second N and P fractions (N_2 and P_2) were isolated. When the procedure was repeated a third time by dissolving the alcoholic precipitate in 75 cc. of dilute alkali, no more of the N fraction separated. Acidification with 16 cc. of 20 per cent acetic acid yielded a trace of a third P fraction (P_3). The clear solution was precipitated again with alcohol and on repeating the entire procedure, neither fractions N nor P separated. The final precipitate, obtained from both alkaline and acidic solutions by addition of alcohol, was designated as fraction C.

With the other solutions treated by this method, significant differences were noted in attempting to exhaust them completely of fractions N and P. With T_L "agar washings" (T.2157 and T.2163), one repetition of the fractionation procedure was sufficient, whereas with T_L broth culture filtrates (T.2145 and T.2146), two or three repetitions were required. Furthermore, the amounts of both N and P fractions were invariably greater in plain broth than in synthetic medium culture filtrates. The P fraction was even completely absent in one batch of synthetic medium culture filtrate (T.2158).

Fraction C always gave positive Molisch and biuret tests. In one experiment, in which particular care was taken to remove fractions N and P completely, the negative biuret reaction observed by Apitz was not confirmed. Since qualitative color tests as indications of chemical

structures must be accepted with considerable reserve, attention is called to the following analytical data. The high nitrogen values consistently found in the various C fractions, *i.e.*, 8.1, 8.5, and 12.4 per cent from filtrates T.2158, T.2157, and T.2145, respectively, suggested the presence of appreciable quantity of protein-like substances. Accordingly, when fraction C of T.2158 was refluxed for $2\frac{1}{2}$ hours in 2 N HCl, only 10.4 per cent reducing substances, calculated as glucose, were formed. For comparison, it may be noted that on hydrolysis, the specific carbohydrates of Types II and III pneumococcus, which do not contain any nitrogen, yield about 70 to 75 per cent reducing substances calculated as glucose; whereas that of Type I pneumococcus, containing 5.8 per cent nitrogen, is hydrolyzed to the extent of 28 per cent (16). It must be emphasized, therefore, that in its present state of purity, fraction C cannot be designated as a carbohydrate.

The N fractions were insoluble at neutral pH and, when tested for activity, were suspended in normal saline. In all cases but one (T.2146), they were only tested for skin-preparatory potency. The solubility of the various P fractions was very irregular. After separation from the main solution by addition of acetic acid, they had a tendency to redissolve in dilute solutions of this reagent. Previous to injection, suspensions of P fractions were cleared by adding N/10 NaOH to neutrality (litmus). When tested undiluted, care was taken to have the solutions practically isotonic. All of the dilutions were made in physiological saline.

A quantitative biological assay of the various filtrates and their fractions is summarized in Table I. The N and P fractions cited in column 2 refer to their respectively pooled preparations (1 + 2 + 3, etc.) isolated from a given bacterial filtrate. In one case (T.2145) several P fractions were tested separately. The solubilities designated for the latter referred to their tendency to redissolve in an excess of dilute acetic acid, as mentioned above. For convenience, the total reacting units, given under column 8, have been calculated to the nearest hundred.

As can be seen from Table I, most of the activity originally present in a bacterial filtrate was lost during the process of fractionation. Even with the best recovery in fraction C of T.2158, the purification

TABLE I

Quantitative Biological Assay of Bacterial Filtrates Fractionally Precipitated with Dilute Alkali, Acid, and 95 Per Cent Alcohol

Filtrate	Fraction	Volume	Dry weight per cc.	N (dry basis)	Titer per cc.		Total reacting units (titer X volume)	Reacting units	
					Skin-preparatory units	Reacting units		Per mg. dry weight	Per mg. nitrogen
T.2158 <i>B. coli</i> grown in synthetic medium	Original	845	19.4	8.70		125±75	106,000	6.5	74
	C	50	71.0	8.14		500±300	25,000	7.1	87
	N	28			0				
T.2163 <i>B. typhosus</i> T _L "agar washings"	Original	170	19.0	12.3		63±38	10,700	3.3	27
	C	51				1.5±0.5	1000		
	P	33				0	0	0	0
T.2157 <i>B. typhosus</i> T _L "agar washings"	Original	165	21.7	12.1		300±200	500,000	13.8	114
	C	102	6.1	8.5	>1	24±22	2500	4.0	46
	N	5.5			0				
	Alcohol supernatant concentrated	70	63.7	5.2		0	0	0	0
T.2145 <i>B. typhosus</i> T _L in veal infusion broth	Original	3350	25.8	12.9		>2	6700+		
	P ₁ (insoluble)	112	1.4	11.0		>1	100+		
	P ₁ (soluble)	112	7.9	13.9		>1	100+		
	P ₂ (insoluble)	112	8.5	13.6		>1	100+		
	C	335	107	12.4		0	0		
T.2146 <i>B. typhosus</i> T _L in veal infusion broth	Original	2720				13±8	35,000		
	C	272			>1	>1	300+		
	N	112				0	0		
	P*	73				>1	100+		
T.2154 <i>B. typhosus</i> T _L in veal infusion broth	Original	2775				>2	6000+		
	Concentrated <i>in vacuo</i> and precipitated with alcohol	280				>2	600+		

N = nitrogen.

* Contaminated with staphylococcus.

was only apparent, since the reacting units per milligram of dry weight and of nitrogen remained practically unchanged. In the last two columns of the table, for example, it is seen that filtrate T.2158 had 6.5 reacting units per mg. of dry weight, whereas in fraction C this had only increased to 7.1; similarly, the activity per milligram of nitrogen had only increased from 74 to 87.

2. *Purification of Active B. typhosus, Meningococcus, and B. coli Culture Filtrates by Means of Dialysis.*—The extent of purification attained by dialysis has been studied quantitatively in the following bacterial filtrates.

T.2156. *B. typhosus*, T_L Strain, "Agar Washings" Filtrate.—Plain agar Kolle flasks were inoculated with a 24 hour T_L broth culture and incubated for 24 hours. The growth was washed with phenolized saline, centrifuged, and filtered twice through a Berkefeld V candle. The final pH was 8.2.

T.2189. *B. typhosus*, T_L Strain, "Agar Washings" Filtrate.—This preparation only differed from T.2156 as follows: The growth was washed with distilled water; three filtrations were required; merthiolate was added to a final concentration of 1:20,000.

T.2165. *B. typhosus*, T_L Strain, Synthetic Medium "Agar Washings" Filtrate.—Kolle flasks were prepared containing the following synthetic medium: NaCl 5 gm., K_2HPO_4 1 gm., KH_2PO_4 1 gm., $MgSO_4$ 0.2 gm., $CaCl_2$ 0.1 gm., Difco neopeptone 20 gm., 1 cc. of 25 per cent sodium lactate, 1 cc. of 10 per cent dextrose, and 20 gm. of agar were dissolved in distilled water and diluted to 1 liter. The pH was 6.4. 24 hour cultures of *B. typhosus*, T_L strain, grown on Kolle flasks prepared as described above, were washed with saline, centrifuged, and resuspended in fresh saline, making a concentration of 600 to 900 million organisms per cc. Fresh Kolle flasks were then inoculated with the washed organisms. After incubation for 24 hours at 37°C. the growth was washed with distilled water (3 cc. per flask), centrifuged, and filtered. The final pH was 7.4.

T.2122. *Meningococcus* 44D. (Group I) "Agar Washings" Filtrate.—Glucose agar flasks, at pH 7.4, were inoculated with a 24 hour culture of *meningococcus* 44D. (group I) and incubated for 24 hours at 37°C. The growth was washed off with phenolized saline (0.4 per cent phenol), centrifuged, and filtered. The filtrate was heated for 45 minutes at 56°C. and refiltered. The final pH was 7.0.

T.2113. *Meningococcus* 44B. (Group III) "Agar Washings" Filtrate.—This filtrate was prepared by the method just described for T.2122.

T.2158. *B. coli* Synthetic Medium Culture Filtrate.³—The preparation of this filtrate has been described above.

T.2159. *B. coli* Synthetic Medium Culture Filtrate.³—A filtrate similar to T.2158 was concentrated *in vacuo* to one-half of its original volume.

³ Prepared in the Lilly Research Laboratories, Indianapolis.

As usually conducted in the laboratory, dialysis is an empirical procedure in which various shapes and kinds of membranes are employed. In order to prevent bacterial contamination, which readily occurs in solutions that are exposed to the air for several days, toluene is frequently added. For the purposes of the present investigation, however, this technique proved inadequate. A simple and reproducible method was developed, therefore, whereby filtrates were dialyzed

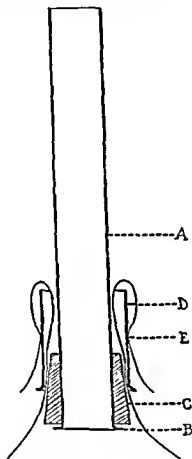


CHART 1. Apparatus for aseptic dialysis.

under strictly aseptic conditions. By means of the apparatus illustrated in Chart 1, the following procedure was successfully adopted.

The bottom of a 150 x 18 mm. pyrex test tube (A) was blown out and the glass melted back to form a rim (B) which served as a support for a rubber stopper (C). A short length of glass tubing (D) was selected to fit loosely over C. (The neck of an Erlenmeyer flask conveniently served for this purpose). A 50 x 50 cm. sheet of plain transparent "Cellophane,"⁴ No. 600, was cut circularly and thoroughly

⁴ We wish to express our thanks to E. I. Du Pont De Nemours and Company, Cellophane Division, New York, for their cooperation in supplying us with various grades of cellophane.

wetted in a large evaporating dish. The membrane was drawn evenly around tube A, by gathering it almost to the top, thus forming a bag. Outer tube D was slipped over and forced down tightly over C. The cellophane was drawn back over D, any excess cut off evenly, and fastened with a copper wire at E. The test tube was plugged with non-absorbent cotton and set into a paper bag which was fastened near the top with a rubber band. The entire apparatus was autoclaved for 20 minutes under 15 pounds of steam and, after cooling, it was ready for use. The solution to be dialyzed was pipetted into the membrane bag. The tube was supported by the clamp of a ring stand kept in the ice chest and immersed in a beaker containing about 600 cc. of distilled water. The solution levels, inside and outside the bag, were maintained as evenly as possible. Every 24 hours for a period of 7 days the beaker in the ice chest was replaced by one containing fresh water. When dialyzing large quantities (more than about 200 cc.) fresh water was replaced twice daily. The fluid was removed with a pipette and the increase in volume noted.

By means of the method described above, more than fifty dialyses have thus far been conducted without any bacterial contaminations. The inconvenient use of toluene, or other antiseptics, has been eliminated. With a larger assembly, using 100 x 100 cm. sheets of cellophane, as much as 1 liter has been successfully handled.

A quantitative biological assay of the various bacterial filtrates, before and after dialysis, is presented in Table II. Before dialysis, the same filtrate preparation was used for both the skin-preparatory and intravenous injections. After dialysis, the fluid removed from the bag was titrated to the end-point for reacting potency in rabbits prepared by single intradermal injections of the original non-dialyzed filtrate. Since the titer of filtrates may change after long periods of time, care was taken to ascertain their potency just previous to dialysis and to titrate the dialyzed solutions as soon as possible thereafter.

It was observed that after dialysis the *B. typhosus* and *B. coli* solutions remained clear, whereas the meningococcus preparations sometimes formed slight precipitates. The decrease of pH, which practically always occurred, suggested the presence of substances whose isoelectric points were on the acid side. The biuret and Molisch tests were usually positive. After removing an aliquot sample for analysis the solutions were adjusted with saline to approximate normality (physiological) and stored in the ice chest. Even after 6 months they had remained clear.

TABLE II
Quantitative Biological Assay of Filtrates from *B. typhosus*, *Meningococcus*, and *B. coli* before and after Dialysis

Filtrate No.	T. 2156/32	T. 2189/47	T. 2165/48	T. 2122/42	T. 2113/31	T. 2158/30	T. 2159/33
Type of bacterial filtrate.....	<i>B. typhosus</i> , plain agar saline washings	<i>B. typhosus</i> , plain agar distilled water washings	<i>B. typhosus</i> , synthetic medium agar distilled water washings	Mg. 44D, glucose agar saline washings	Mg. 44B, glucose agar saline washings	<i>B. coli</i> , synthetic medium (Lilly)	<i>B. coli</i> , synthetic medium concentrated one-half (Lilly)
Before dialysis							
Volume, cc.....	15	36	20	10	15	40	50
Dry weight per cc., mg.....	22.6	18.2	15.8	—	31.5	19.4	38.2
Total dry weight, mg.....	340	655	316	—	473	776	1910
Percentage nitrogen (dry basis)	11.2	12.0	7.9	—	9.4	8.7	10.1
Total nitrogen, mg.	38.0	78.5	25.0	—	44.5	67.5	193
Total nitrogen per cc.....	150±50	150±50	50±	1100±400	2000±500	150±50	125±75
Total units	2250	5400	1000	11,000	30,000	6000	6250
Units activity per mg. dry weight.	7	8	3	—	63	8	3
Units activity per mg. nitrogen.....	59	69	40	—	675	89	32
pH.	7.8	8.5	7.5	7.3	7.3	5.8	6.3
After dialysis							
Volume, cc.....	30	79	35	22	25	49	83
Dry weight per cc., mg.....	4.9	3.9	3.0	—	6.7	1.1	3.1
Total dry weight, mg.....	147	308	105	—	168	54	257
Percentage nitrogen (dry basis).....	14.1	11.5	9.6	—	13.0	13.8	11.0
Total nitrogen, mg.....	20.8	35.4	10.1	—	21.8	7.5	28.3
Titer units per cc.....	100±	116±25	29±	300±167	1000±	75±25	30±20
Total units.....	3000	9170	1010	6600	25,000	3680	2490
Units activity per mg. dry weight... ..	20	30	10	—	149	68	10
Units activity per mg. nitrogen.....	145	256	101	—	1145	491	88
pH.	6.3	7.2	5.8	6.3	5.3	7.3	6.8
Yield, recovery of total units, per cent.....	133±44	170±57	101±	60±34	83±21	61±21	40±27

As can be seen from Table II, the recovery of reacting units, considering the limits of error involved in the biological titrations, has been quantitative. In several cases (T.2158, T.2156, and T.2189), the recovery of skin-preparatory potency was similarly investigated. Here too, it was observed that the recovery of activity was quantitative.

The extent of purification attained by means of dialysis has been summarized in Table III, the data of which were calculated from Table II. As can be seen from Table III, *B. coli* grown in fluid synthetic medium (T.2158/30) was most readily purified; thus, the decrease in total dry weight and nitrogen, 93 and 89 per cent respectively, was greater than in any of the other filtrates. Similarly, the increase

TABLE III

Summary of Purification of Bacterial Filtrates by Means of Dialysis

Filtrate No.....	<i>B. ty.</i> T. 2156 32	<i>B. ty.</i> T. 2189 47	<i>B. ty.</i> T. 2165 48	Mg. T. 2113 31	<i>B. coli</i> T. 2158 30	<i>B. coli</i> T. 2159 33
Percentage decrease in total dry weight.....	57	53	67	64	93	87
Percentage decrease in total nitrogen.....	45	55	60	51	89	85
Percentage increase in nitrogen content (dry basis)...	26	-4	22	38	59	9
Percentage increase in units activity per mg. dry weight.....	186	275	234	136	750	234
Percentage increase in units activity per mg. nitrogen.....	146	271	152	70	452	175

B. ty. = *B. typhosus*; Mg. = meningococcus.

in activity per milligram of dry weight and of nitrogen, 750 and 452 per cent respectively, was also the highest. When a similar preparation was concentrated *in vacuo* to one-half of its original volume (*B. coli* T.2159/33) approximately the same amount of total solids and nitrogen was removed by dialysis, but the increase in activity was only 234 and 175 per cent, respectively. Apparently, therefore, the concentration *in vacuo* may considerably reduce the extent of purification. With *B. typhosus*, a slightly greater removal of total solids and nitrogen was obtained in filtrates of cultures on synthetic medium agar (T.2165/48) than on veal infusion agar (T.2156/32 and T.2189/47). Although the actual reacting titers of meningococcus culture filtrates were decidedly the highest, both before and after dialysis (Table II),

the extent of purification attained by dialyzing T.2113/31 was lower than in any of the other filtrates. Thus, the percentage increase in activity in this filtrate was only 70 per cent, per mg. of nitrogen, and 136 per cent, per mg. of dry weight. Possibly, the use of 1 per cent rabbit blood in the inoculum (17), carried over into the washings, accounts in part for the relatively lower figures. It should be noted, however, that the highest number of reacting units per milligram of nitrogen and dry weight (Table II, 1145 and 149, respectively) was observed in the dialyzed meningococcus filtrate (T.2113/31). By calculating the reciprocals of these values, it may be seen that 0.0067 mg. of total solids and 0.00087 mg. of nitrogen are capable of eliciting the reaction in prepared skin sites at the end-point. The mean figure of the last two horizontal entries of Table III indicates approximately a threefold increase in activity.

DISCUSSION AND CONCLUSIONS

Previous investigations (18) demonstrating the antigenicity of the active principles of the phenomenon under discussion suggested that they were either protein in nature or perhaps associated with proteins.

The recent observations of Apitz (13), however, seemed inconsistent with this point of view, since he reported the isolation from "agar washings" filtrates of a biuret-negative fraction (C) which was slowly dialyzed through cellophane. He further pointed out, incidentally, that the so called nucleoprotein fraction (P) was always active in the phenomenon and apparently arose from bacterial autolysis or extraction. The source of the alcohol-precipitated fraction (C), on the other hand, appeared to result more from active bacterial growth, and although it gave rise to some of the strongest reactions observed, nevertheless it was very labile in character. In all of his experiments, activity was measured by skin-preparatory potency only.

In the present investigation, a quantitative biological assay has been made of bacterial filtrates fractionated in the manner used by Apitz. By accurately titrating the reacting factors of the phenomenon, it was found that, in agreement with Apitz: (a) the alkaline-insoluble N fractions were always inactive; (b) the nucleoprotein P fractions were practically always active; (c) the supernatant alcoholic solutions, after concentration *in vacuo* were inactive; and (d) the

alcohol-precipitated C fractions were highly potent but frequently labile, in one instance being completely inactivated during preparation. The isolation of a biuret-negative C fraction, however, has not been confirmed. The nitrogen values for the latter fractions, furthermore, were very high, ranging from about 8 to 12 per cent in different preparations. By titrating the recovery of reacting factors, it has been found that most of the activity originally present in a bacterial filtrate was lost during these procedures of concentration. Even with the best recoveries observed, the extent of purification attained was insignificant when the activity per milligram of dry weight or of nitrogen was measured.

An extensive investigation of the dialysis of various bacterial filtrates proved that this operation effected excellent purification of their active principles. A convenient and simple apparatus was developed whereby dialysis through cellophane membranes was readily conducted under strictly aseptic conditions. With this technique, the increase in activity per milligram of dry weight ranged, in the preparations studied, from 136 to 750 per cent; similarly, the purification with respect to nitrogen varied from 70 to 452 per cent in the filtrates which were quantitatively assayed. The retention of these active principles by cellophane membranes confirms the former observations of Shwartzman (1), Gratia and Linz (19), and Plaut (20) and, furthermore, is in accord with their antigenicity.

SUMMARY

A quantitative biological assay of the products obtained from the dialysis of *B. typhosus*, meningococcus, and *B. coli* culture filtrates has been undertaken. It was found that the active principles of the phenomenon of local skin reactivity to bacterial filtrates were retained by cellophane membranes. An appreciable purification was thus effected, amounting, on the average, to about a threefold increase in reacting potency per milligram of dry weight and of nitrogen.

Attempts to purify bacterial filtrates by the fractional precipitation of their concentrates with dilute alkali, acid, and alcohol were unsuccessful.

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ANAPHYLACTIC SENSITIZATION WITH CHEMICALLY DEFINITE COMPOUNDS

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The investigations of Landsteiner and others have demonstrated that in anaphylaxis the significance ascribed to proteins has to be reconsidered. The component part of the antigen responsible for specificity need not necessarily be a protein but may be a chemically definite simple compound (hapten). The haptens react *in vitro* with antibodies, and can, under certain circumstances, prevent anaphylactic reaction with the full antigen (inhibition reaction (1)) (Jadassohn and Schaaf (2)). Landsteiner (3) also succeeded in inducing anaphylactic shock by means of chemically definite compounds (resorcinoldisazo-*p*-succinanilic acid and resorcinoldisazo-*p*-suberanilic acid) in appropriately sensitized animals. It seems, however, that the protein component plays an important part in inducing anaphylactic shock; usually the chemically definite compound does not suffice, but must be coupled with some protein.

Since haptens alone do not usually suffice to sensitize, it is generally understood that proteins, serving as carriers, play an important rôle in sensitization, the so called full antigen (hapten + protein) being necessary. For clinical medicine, especially for dermatology, the establishment of these facts presents a problem. We are inclined to conceive certain forms of skin hypersensitiveness, especially of the urticarial type, as anaphylactic (Jadassohn). However, not only proteins can induce such reactions but, and this is of special importance, sensitization can often be produced also by protein-free compounds of known chemical constitution (drugs). As early as 1907 Wolff-Eisner (4) evolved a hypothesis to clarify this problem by assuming that the protein molecules couple with the drug within the

organism, thus producing a full antigen and causing hypersensitivity. Obermayer and Pick (5) had already ascertained that proteins derived from the same species would become heterologous (*koerperfremd*) by iodination, nitration or diazotization, and these facts Wolff-Eisner had adduced as a corroboration of his hypothesis. Proteins altered in this manner induce in the animal organism the same reactions as if heterologous protein had been injected. Landsteiner found later that azoproteins can induce in rabbits the formation of antibodies which react *in vitro* specifically with the azo component (hapten), even if they had been prepared with serum from the same species.

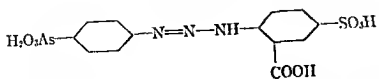
EXPERIMENTAL

Jadassohn and Schaaf were in some cases able to sensitize guinea pigs by means of diazotized atoxyl coupled with guinea pig serum, *i.e.* without heterologous protein. These experiments have not as yet been published in detail but have been briefly mentioned (6). One of these tests follows below.

Guinea Pig 16-63.—On the 1st, 7th, 14th and 21st day intraperitoneal injections of 1.5 cc. diazotized atoxyl coupled with guinea pig serum (prepared according to Landsteiner) were given; on the 49th day the Schultz-Dale test was made (7).

The curve in Fig. 1 clearly demonstrates that the animal treated previously with diazotized atoxyl coupled with guinea pig serum has been sensitized to this substance. No heterologous protein had been used, either for the sensitization or for eliciting the anaphylactic response.

Undoubtedly all these facts seem in good agreement with Wolff-Eisner's hypothesis. There still remains the important point of actual proof that within the organism the chemically definite compound couples with the body protein and that such a coupling product of the organism itself is then able to sensitize. The following experiments appear to confirm this idea. The sodium salt of atoxyl-diazo-amino-sulfoanthranilic acid (2-carboxy-4-sulfodiazoaminobenzene-4-arsenic acid),



was used for this purpose.

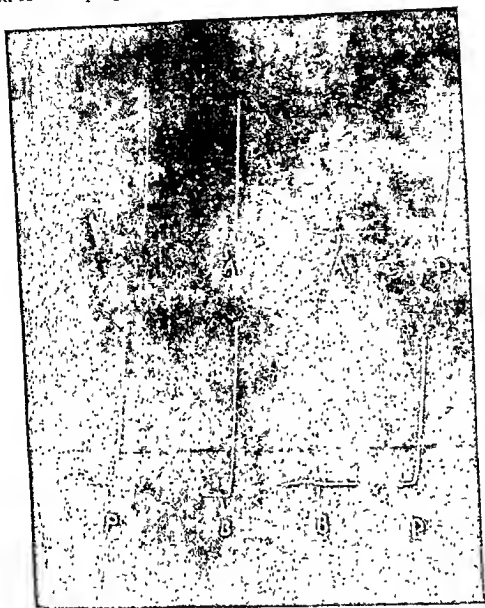


FIG. 1. Above, left horn; below, right horn. A = 0.4 cc. of 1 per cent guinea pig serum-azoprotein. B = 0.4 cc. of 1 per cent rabbit serum-azoprotein. P = 1 cc. pituglandol 1:250. x = rinsing.

Preparation and Properties.—5.86 gm. atoxyl dissolved in 30 cc. N/1 HCl and cooled to about 0°C. were diazotized with 20 cc. of N/1 nitrite solution. The resulting diazo compound was slowly stirred into a neutral solution of 4.6 gm. 6-amino-3-sulfobenzoic acid plus 6 gm. sodium acetate in 150 cc. of water, the temperature being kept at about 6°C. The solution was stirred for a short time

and finally neutralized with Na_2CO_3 . After the coupling was completed, the solution was heated for a short time to 50°C ., then 50 gm. of pure sodium chloride were added and the mixture kept at 0° . Soon the diazoamino compound sepa-



FIG. 2. Guinea pig 4-54, previously treated with sodium atoxyl-diazoamino-sulfoanthranilate. Schultz-Dale experiment on the 54th day.

Above, left horn; below, right horn. A.H. = 10 mg. atoxyl-azo-chicken serum. B = 50 mg. sodium atoxyl-diazoamino-sulfoanthranilate. P = 1 cc. pituglandol 1:250. x = rinsing.

rated from the solution in fine yellow crystals. After two recrystallizations from water the substance was dried *in vacuo* at 50° .

Sodium atoxyl-diazoamino-sulfoanthranilate dissolves readily in water to give

a yellow solution. In the dry state and in alkaline solution it is quite stable. On acidification of an aqueous solution with acetic acid the diazonium salt of *p*-arsanilic acid is liberated. This compound is then able to couple with appropriate substances to form real azodyes and also with proteins to yield colored azo-proteins.

Preparatory Treatment of Guinea Pigs.—A 1 per cent solution of sodium atoxyl-diazoamino-sulfoanthranilate in physiological sodium chloride solution was injected intraperitoneally at weekly intervals for 4 weeks. The first three injections were 2 cc. each, the last injection 4 cc. 52 to 88 days after the injections the Schultz-Dale test was performed (7). Of the 13 treated guinea pigs 3 had died; the remainder were tested. 5 of these were also tested for an anaphylactic reaction with the sodium atoxyl-diazoamino-sulfoanthranilate which had been used in the preliminary treatment and all of them were tested for anaphylactic reaction with diazotized atoxyl coupled with chicken serum (prepared according to Landsteiner).

RESULTS

1. None of the 5 animals gave an anaphylactic reaction with sodium atoxyl-diazoamino-sulfoanthranilate (10–100 mg. per 50 cc. bath solution).

2. All 10 animals responded to the atoxyl azoprotein with an anaphylactic reaction (contraction of the uterus with 2.5–10 mg. (in 50 cc. bath solution) which upon repeated contact did not reappear (neutralization).

3. By sodium atoxyl-diazoamino-sulfoanthranilate the subsequent reaction with the azoprotein was, with one exception, not interfered with. These and other experiments on neutralization will be discussed later.

To illustrate these experiments, a curve is reproduced in Fig. 2.

DISCUSSION

Sodium atoxyl-diazoamino-sulfoanthranilate injected into guinea pigs sensitizes the animals not to this compound but to an azoprotein corresponding to it. At present, for this behavior there seems to be only one satisfactory explanation. The diazo compound formed in the organism from the sodium atoxyl-diazoamino-sulfoanthranilate used in the preliminary treatment recouples to produce an azoprotein which sensitizes. The Schultz-Dale test clearly indicates this azoprotein hypersensitiveness. By itself, the chemically definite compound produces no reaction in the Schultz-Dale test, probably because under

the conditions of the test formation of azoprotein in the uterus occurs not quickly enough or not at all.

We therefore have reached the following conclusions. Chemically definite compounds can sensitize animals in the same way as chemically definite compounds (drugs) can sensitize human beings. In this process coupling of the chemically definite compound with the body protein takes place and it is the coupled product which sensitizes. The hypersensitiveness caused in this manner cannot be demonstrated with the chemically definite compound used in the preliminary treatment but only by an anaphylactic reaction with the corresponding azoprotein. The proof of the formation of this azoprotein *in vivo*, however, makes it quite likely that chemically definite compounds not only can produce anaphylactic hypersensitiveness but that, in conjunction with the body protein, they can also induce in the organism anaphylactic reaction. For at least some cases this is a confirmation of the Wolff-Eisner hypothesis. The proof that sensitization is possible without the use of heterologous protein is, however, also of importance for the whole conception of anaphylaxis. Especially striking is the fact that a non-anaphylactogenic substance can be transmuted by the organism itself into an anaphylactogenic compound and that the body protein plays an important part in this process.

SUMMARY

Injection of sodium atoxyl-diazoamino-sulfoanthranilate into guinea pigs produces an anaphylactic hypersensitiveness to the corresponding azoprotein (Schultz-Dale test). This leads to the conclusion that the injected sodium atoxyl-diazoamino-sulfoanthranilate first decomposes and then couples *in vivo* with the body protein to form the corresponding azoprotein and that therefore it is this compound, produced within the organism itself, which sensitizes.

The authors are greatly indebted to the Schweizerische Volkswirtschaftsstiftung and to Mrs. Bruno Bloch for financial assistance enabling them to carry out these investigations.

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CHANGES IN THE CUTANEOUS LYMPHATICS OF HUMAN BEINGS AND IN THE LYMPH FLOW UNDER NORMAL AND PATHOLOGICAL CONDITIONS

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PLATES 11 TO 13

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Earlier work (1) has shown that intradermal injections of vital dyes are to a large extent intralymphatic, for dye enters the lymphatics where they are torn or ruptured by the injecting needle. It renders the rich superficial plexus visible and within a few minutes some of it passes into the larger, subcutaneous, draining lymphatics and these appear now through the skin like colored streamers. The existence of a significant cutaneous lymph flow as thus evidenced has suggested the present study of the variations in flow under physiological and pathological conditions.

It was necessary to ascertain first of all whether intradermal injections of dye would yield evidence of changes occurring in the character of the lymph flow. A series of tests directed to this end showed the method to be adequate to demonstrate those variations in lymph flow which are known to occur. Long and intensely colored streamers appeared in the skin of limbs heated or actively moved and short, lightly colored streamers in limbs at rest, conditions known to increase or decrease the flow of lymph respectively (2-4). The technique was next employed to detect changes in the character of the lymph flow under conditions having an unpredictable influence upon it.

Method

In the previous work (1), demonstrating lymphatic capillaries in human skin, 0.05 to 0.1 cc. of an 11 per cent aqueous isotonic solution of a vital dye, patent blue V,¹ was injected intradermally in the forearms of healthy volunteers. These

¹ General Dyestuffs Corporation, New York.

relatively large injections led to the formation of colored streamers in normal arms, which extended so rapidly to the axilla that one could hardly employ the rate at which they formed to detect increases in lymph flow. In the present work, desiring to distinguish between increase and decrease in streamer formation under differing physiological conditions, we have resorted to much smaller injections of diluted isotonic dye solution which produced short, pale streamers in normal skin, that increased or decreased in length or intensity when conditions were varied. A mixture of equal parts of Locke's solution and the aqueous isotonic 11 per cent solution of patent blue V was employed in all the work and the resulting 5.5 per cent solution, autoclaved, was injected intradermally during a period of 45 seconds to 1 minute in amounts ranging from 0.01 to 0.04 cc. Thus the concentration of the dye was but half of that used in the previous work and the amount of pigment introduced but 1/10 to 2/10 the previous quantity.

Luer tipped, glass and metal 1 cc. record tuberculin syringes served for all the injections. They were graduated upon the handle of the plunger so that accurate measurements could be made, because the intensity of the color of the dye in the barrel obscured the markings there. To obtain exceedingly superficial blebs of dye, thus insuring injection of the superficial plexus of lymphatic capillaries, No. 30 gauge platinum iridium needles were used. Unless otherwise stated injections into the forearm were made while the subjects sat with it horizontal, comfortably supported upon a table at the level of the apex beat of the heart. Injections into the skin of the ankle were made while the subjects sat or reclined with both legs supported horizontally. Before each test the subject remained at rest 30 minutes or longer. Similar injections were then symmetrically placed in both limbs, one of which remained at rest while the other was subjected to the test procedure. When this was not practicable, the control injection was first made, and its effects observed for half an hour, after which the test injection was given.

For the photographs a steel gallows was used with an extensible horizontal arm 40 cm. in length supporting at its outer end a universal joint carrying a standard leica camera (f. 3.5 lens) and lighting apparatus. A binocular microscope was similarly arranged on a long metal arm. Both camera and microscope could be tilted at any angle and extended over a hospital bed when studies were to be made upon patients, and the results of the injections were simultaneously observed and photographed. The camera carried on its face a 1 to 1 reproduction device for taking pictures at natural size, and the device and frame were of such length that when the instrument was placed a millimeter or two above the skin surface the focal point of the lens was situated a millimeter or more deep in the skin. A photoflood bulb and a series of 8 inch condensing lenses throwing a beam of light 2.0 inches in diameter at the focal point of the camera gave a brilliant and constant illumination. The natural size photographs were taken at exposures of 1/20 of a second, and at intervals of 5 seconds when desired, using supersensitive panchromatic film and a red gelatine filter,² to bring out the blue color of the dye. To record

² An Eastman Kodak Co. No. 25 gelatine film light filter.

streamers following the injection photographs were taken at regular intervals during 20 minutes at a distance of 40 cm. A single photoflood bulb and reflector afforded sufficient light for 1/10 second exposures. Tracings of the dye streamers on a piece of curved celluloid held over the limb, were made with patent blue V instead of ink, the dye giving far better results than the latter when used with the finest drawing pens.

As a matter of routine the appearance of the dye in the lymphatics and the behavior of their colored contents was noted, as was too the state of dilatation or contraction of the channels, the ease or difficulty with which dye entered them, the distance it extended in them when first injected, and the extent to which the superficial plexus was filled at that time. For example, only part of the dye injected into normal skin finds its way into the lymphatics directly, much remaining as an interstitial bleb at the point of injection. In other circumstances, as will be seen in the following paper, almost all the dye enters the channels directly and is carried farther in them. We observed also the rate of dye escape from the lymphatics, as evidencing changes in their permeability, and further, the extent of interstitial spread of color and the rapidity of its disappearance. The speed and intensity of streamer formation was watched for a period of half an hour, as evidence of the rate of lymph flow. At hourly intervals or oftener, for 5 hours, observations were made of the increase or decrease in the intensity of the streamers with their final disappearance perhaps, together with the rate of paling of the dye bleb, or its interstitial spread.

The Behavior of Dye Solutions in Lymphatic Capillaries of Normal Skin

The events which follow an injection of dye on the volar surface of the forearm have been described in an earlier paper (1). In the present work, as mentioned above, much smaller amounts of dye were used in order to produce short streamers which varied in length or intensity according to the local conditions. Natural size photographs of typical injections in the skin of the volar surface of the normal forearm are shown in Figs. 1 and 2 as a standard wherewith later findings are to be compared. In each figure the first four exposures were taken at intervals of 15 seconds after beginning an injection of approximately 0.02 cc. of dye solution, the fifth and sixth at intervals of 30 seconds, the seventh and eighth after 3 and 4 minutes and the last at the 20th minute.

The figures show dye directly entering the network of superficial lymphatics (through channels torn by the needle) and spreading out within them. At the point of the injecting needle much dye remains as an interstitial bleb. In all instances, during the injection and for

some time thereafter, the colored fluid continues to spread through the superficial lymphatics, increasing the area of the injected region. Diffusion occurs, blurring the borders of these channels, where before the outlines had been distinct (Figs. 1 *e* to *h*; Figs. 2 *d* to *h*). In a few minutes the injected region may show one or more pseudopods of color, extensions of dye in subcutaneous lymphatics. As the lymph flow carries the dye further from the injection site, they become visible through the skin as colored streamers, which, even in a resting arm, may extend 10 or 15 cm. in 20 minutes.

In the skin of the ankle the lymphatic plexus seems less rich, the channels smaller and lymph flow slower. In the following paper, photographs of the results of a dye injection in the normal ankle are compared with those of similar injections in the skin of edematous ankles.

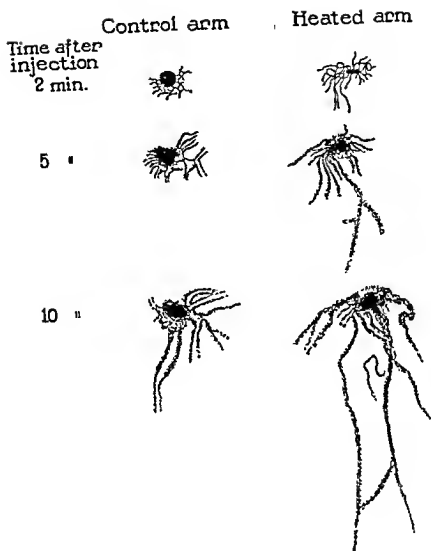
The Effect of Agents Known to Stimulate Lymph Flow

It is common knowledge that lymph flow is increased by applications of heat (2, 4-6), by massage (4, 7-9), by activity (3, 7, 10-13) or by hyperemia (5, 6), and that it is diminished in limbs that are at rest (4, 7, 9, 14).

The Influence of Heat.—We injected patent blue V into the skin of the volar surface of both arms of 8 normal individuals, subjecting one arm to the influence of heat while the other remained at room temperature.

During a 45 minute rest period prior to the test the subjects sat with both forearms lying on the table, as already described. One arm was then gently submerged in a large cauldron filled with water maintained at 46-47°C. while the control arm rested, in the same relative position, either upon the table, supported at wrist and elbow by wooden blocks, or submerged in a large basin of water at room temperature. After variable periods of immersion, 5 to 15 minutes, 0.02 to 0.03 cc. of the dye mixture was injected into the volar surface of the control forearm about one-third of the way from the wrist to the elbow. As soon as possible thereafter, a similar injection was placed in the submerged arm while it was still under water. In a few instances the arm was lifted out, injected and at once resubmerged. In alternate tests the warmed arm was injected first. In all of them the arm remained just covered by the hot water where the effect of the injections could be observed through the binocular microscope and tracings made on celluloid. Photography was not attempted in these instances.

In every case hyperemia developed in the immersed arm. The lymphatic capillaries disclosed by injection seemed slightly wider than in the control arm.



TEXT-FIG. 1. The effect of warmth on lymph flow. Tracings of 2 similar intradermal injections of dye in the forearm, 2, 5 and 10 minutes after injecting. Dye escape from the lymphatics is indicated by the stippling. Column 1 shows the result in the normal resting arm, column 2 in an arm resting in warm water at 46-47°C. In the latter the streamers developed more rapidly, the interstitial bleb of dye was smaller. 1/2 natural size.

Dye entered them much more readily than was the case in normal skin and seemed to be distributed farther in them. As a result, the area of lymphatic capillaries injected occupied a wider circumference, the network seemed more richly injected, and the amount of dye remaining as an interstitial bleb was far less. Dye escape from such capillaries was rapid too and colored streamers, more intense than in the control arms, appeared earlier and became longer. Text-fig. 1, a 1/2 natural size reproduction of the celluloid tracings of a typical test, shows the behavior of the colored streamers. As indicated, the tracings were made 2, 5 and 10 minutes after the beginning of the injections in the two arms. Each injection required about 1 minute.

The findings show clearly that the application of heat increases the size and intensity of the colored streamers of dye flowing away in the subcutaneous lymphatics from a localized region of injection. The changes are such as one would expect with increased lymph flow.

The Effect of Muscular Movement.—In 8 tests on 4 normal persons approximately 0.02 cc. of patent blue solution was injected intradermally on the volar surface of both forearms, following the rest period as usual. For 20 minutes thereafter the muscles of one arm were contracted and relaxed by clenching the fist at intervals of 3 to 5 seconds and making punching movements every 10th second, drawing the exercised arm back to the chest at the same level as the control arm. Occasional pauses were allowed for the inspection of the injected area under the binocular microscope and to make tracings.

In all the tests dye escaped more rapidly from the lymphatic capillaries of the exercised arms and diffusion of dye into the tissues was also greater. Dye streamers developed sooner, were broader, longer, more numerous and more deeply colored. In some instances streamers only twice as long as those in the resting arm developed in the 20 minute period, that is to say streamers about 25 cm. long. In other instances deeply colored bands reached the axilla with excessive speed, in 3 or 4 minutes. The results were too irregular to show in a single typical tracing or photograph, yet always the streamers were greater in the exercised limb.

Similar injections were made in the skin of both ankles of the same 4 volunteers, first in one leg which was allowed to rest horizontally for 20 minutes and then in the other, after which the subjects walked shoeless about the laboratory. The activity brought about changes in the dye movement, long streamers often reaching above the knee in 5 to 10 minutes, when walking was permitted, whereas they had extended only 3 to 7 cm. after 20 minutes in the limbs at rest. Once again a procedure increasing lymph flow brought about enhancement of the movement within lymphatics.

Effects of Massage.—In 7 tests the limb was massaged after an intradermal dye injection in either the ankle or forearm. When the area injected was massaged immediately, the colored fluid extended 1 or 3 cm. farther into the superficial lymphatic capillaries than in the skin on the control side, and longer and more brilliant streamers appeared earlier. These streamers were much darker than in

our previous instances. Although it is well known that massage produces an increase in lymph flow from cannulated lymphatics (4, 7, 9), the rapid appearance of colored streamers can be taken only as evidence of a movement of the dye within the channels, not of a flow of lymph. Manipulation of the limb may have squeezed dye into the opened lymphatics and forced it along them. The intensity of the color of the streamers showed that the dye had been diluted less than usual. It is of interest to note, in connection with other tests reported in the accompanying paper, that retrograde movement of colored fluid within the lymphatics did not occur, save for a distance of 1 or 2 cm. and then only within the superficial lymphatic capillaries.

In 6 other tests dye injections were made 5 cm. below the antecubital fossae of both arms, and the lower arm, wrist and hand were massaged carefully, avoiding manipulation or pressure upon the injected area. In all these instances too, dye streamers extending from the sites of injection in the massaged limbs were longer and more deeply colored than was the case in the controls. The differences were like those shown in Text-fig. 1, but varied much from instance to instance. In these tests as in those just described, much pressure must have been transmitted to the injected area.

The Effects of Passive Movement, Suction and Posture

The findings reported to this point have all shown that procedures which are known to increase lymph flow caused an enhancement in the size, number and intensity of the colored streamers developing after injection of small amounts of dye in the skin. In resting limbs, in which presumably lymph flow was least, that is to say in the control arms, streamer formation was least. With this much ascertained, we next studied the movement of dye in the skin lymphatics of limbs subjected to passive movement, to suction and to changes in posture, all of which must influence lymph flow in the intact limb in ways which though not definitely known are reasonably predictable.

The effects of passive movement were found to be far less pronounced than those of active movement. 4 tests were carried out, on 2 normal individuals. A brief summary of one will suffice.

Both arms were allowed to lie at rest on the desk for half an hour, after which 0.02 to 0.03 cc. of dye was injected, either on both forearms as usual or, in 2 tests, over the biceps muscle. At once one wrist was placed in a sling attached to the rim of a wooden wheel a foot and a half in diameter, placed at the subject's side, a little in advance of him and with its hub at the level of the control arm. A motor revolved the wheel once each second for a period of 20 minutes. With the wrist resting in the sling in mid-pronation and supination a movement was imparted to

the arm not unlike the punching motions used in studying the effects of active movement. During the movement every attempt was made to keep the muscles as relaxed as possible. Movement was stopped at the end of the 3rd, 7th, 10th, 15th and 20th minutes to make rapid tracings of the dye streamers.

The tests gave evidence of definite differences in the spread of dye in the two arms but these were relatively slight. In each instance the streamers in the test arm were less than twice as long as those on the control side, which averaged 10 cm. after 20 minutes.

Suction Increases the Lymphatic Drainage of Dye Injected into Living Skin.—Earlier work (1) has shown that dyes and other foreign substances gain entrance into the skin lymphatics surrounding a cut or scratch. Immediate pressure or massage over an injected area of skin drives colored fluid into the lymphatics. The time honored custom of sucking a cut or scratch presents a question: What happens under these circumstances to the foreign material which has entered the superficial lymphatics? A few injections were done to test the point.

Small injections of dye were made in the usual way in both arms of normal subjects, one to serve as a control, the other to be sucked as would be done by an individual pricked or cut in every day life. Under the binocular the spread of dye in the lymphatics was observed and subsequent streamer formation followed as usual.

The act of sucking not only failed to remove all the dye but forced it into the lymphatic capillaries draining the injected area. In 3 to 5 minutes streamers 4 to 6 cm. long appeared while in the control arms no streamers developed in so short a time. Dye escaped into the tissues in larger amounts, judging from the intensity of the secondary diffuse staining about the injected lymphatics, and subsequent lymphatic drainage was also greater, for streamers appeared earlier and were longer and more intensely colored than in the control arm. 15 or 20 minutes later dye could be seen 20 cm. or more above the site of injection as in arms which had been massaged. In the control arms the dye had moved but 8 or 10 cm.

The test was repeated three times with similar results.

The Effects of Posture on Lymph Flow.—Changes in posture brought about great variations in the movement of dye in the lymphatics, as judged by the character of streamer formation.

In 12 trials a subject was seated with one arm resting as usual on a desk, the forearm in supination and one leg propped horizontally on a chair of the height of the one in which the individual sat. Limbs placed in this way served as controls.

The subject's other arm hung downward and the other foot rested on the floor. After a preliminary period of half an hour 0.02 to 0.03 cc. of dye solution was injected intradermally on the anterior surface of each ankle at the level of the malleoli of the tibia and on the volar surface of each forearm about 1/3 the way between the wrist and elbow, keeping the relative positions of the limbs constant for 20 minutes thereafter. At the end of this period each dependent limb was raised to a level far above that of its fellow, the test arm being raised vertically from the shoulder and the test leg propped on the desk at the level of the lower ribs or by means of blocks at a level well above the shoulder.

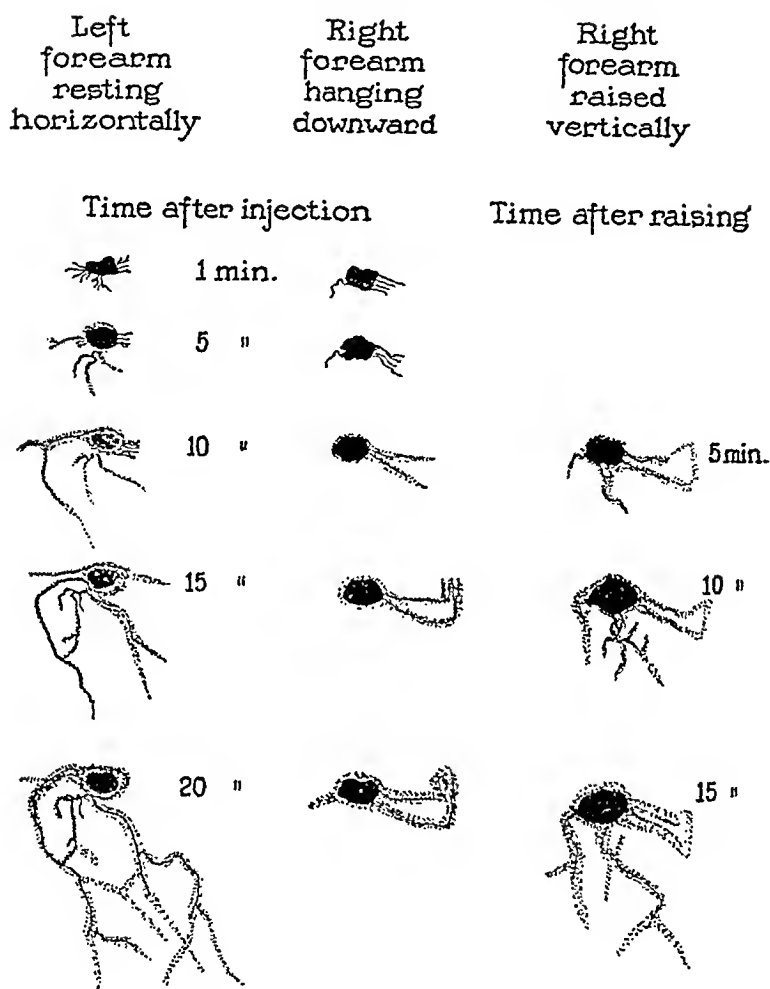
In another 8 instances one leg or arm, after both had remained horizontal or hanging directly downward for 20 minutes, was raised and at once injected, leaving the control limb, also injected at nearly the same time, in the original position. The new posture was maintained for another 20 minutes during which streamer formation was noted. Finally the control limb was also raised and observed for any further extension of its dye streamers.

In Text-fig. 2, tracings are reproduced from a test of the first type. In column 1 the dye spread in the control arm is depicted, in tracings taken as soon as possible after making the injection and at 5 minute intervals thereafter. In column 2 the condition in the dependently hanging arm is shown at the same time intervals. It will be seen that only a few channels running transversely were filled with dye. After 20 minutes this arm was raised vertically over the head, and column 3 shows the tracings of streamers from the same untouched injection site at intervals of 5 minutes thereafter.

Invariably elevation of a dependently hanging limb to a horizontal position, or better to a vertical position, led to extension of dye streamers and frequently to a formation of new ones. Their formation was greatest when a limb which had been hanging downward was raised and then injected immediately. They were less prominent when the limb previously held in a horizontal position was raised vertically and injected, and least marked when a dependently hanging limb was injected and 20 minutes later raised and no further injection made, as in Text-fig. 2. Presumably in such instances much of the dye had already escaped from the lymphatics into the interstitial tissues and hence was no longer carried along rapidly.

The Movement of Lymph Following Release of Lymphatic and Venous Obstruction

The changes in lymph flow in a limb just raised from a dependent position may be regarded as the result of a release of partial lymphatic and venous obstruction. Fluid accumulates in a dependent limb



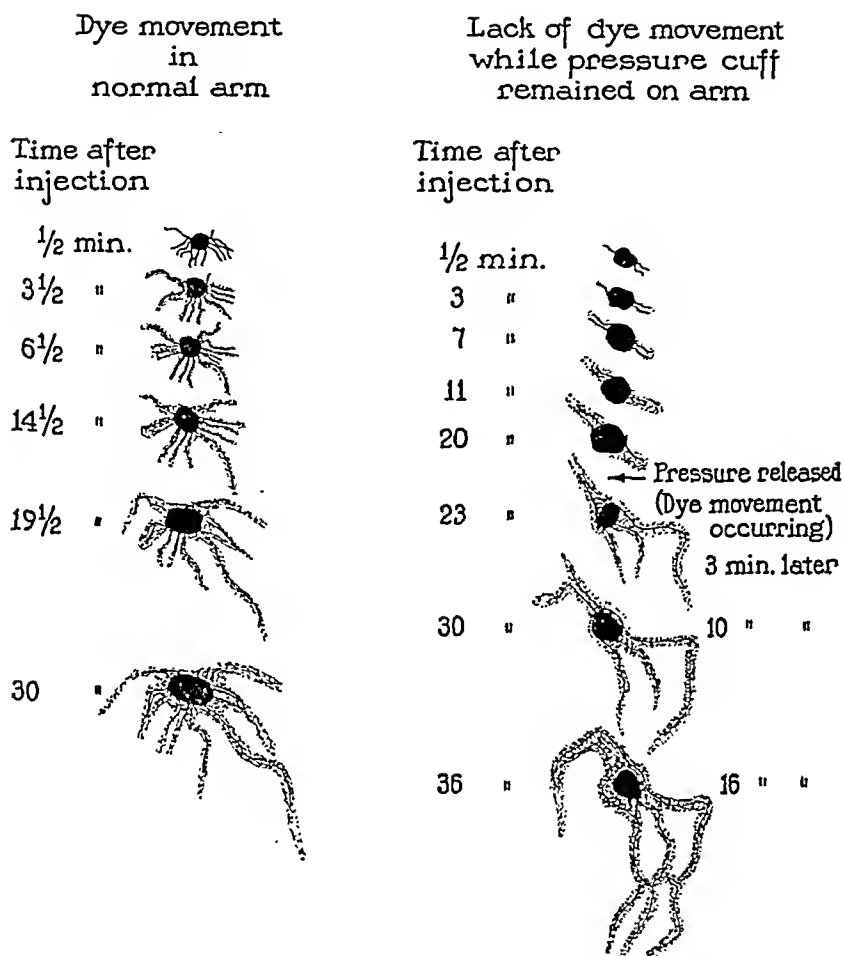
TEXT-FIG. 2. The effect of posture upon lymph flow. Tracings of 2 similar intradermal injections of dye in the skin of the volar surface of normal forearms, drawn as soon as possible after the injections and at 5 minute intervals thereafter. The subject sat with one arm resting on a desk, the forearm in supination and the other arm hanging downward. The left column shows the spread of dye in the skin of the forearm resting horizontally, the center column the behavior of the dye in the dependently hanging arm. After the fifth tracing this arm was raised vertically above the subject's head. Three more tracings at 5 minute intervals show the influence of change in posture upon the dye movement. $1/2$ natural size.

(15-19), and in this posture lymph flows more freely than usual from a lymphatic cannulated in the dependent region (7). Starling first showed an increased production of thoracic duct lymph during a rise in abdominal venous pressure (2, 14), and White, Field and Drinker (7) have reported similar effects of peripheral venous obstruction upon subcutaneous lymph. More will be said of this below, but in all the experiments mentioned lymph flow has been measured from opened cannulated lymphatics no longer subjected to the hydrostatic pressure of the column of lymph above the point of cannulation. In the intact resting dependent limb lymph does *not* flow, as our tests have just shown.

What can be said of the movement of fluid in the closed lymphatic system during and after periods of lymphatic obstruction and of partial and complete venous obstruction? Upon release of a temporary venous or arterial occlusion there follows an intense reactive hyperemia. What effect does this have upon lymph flow? An attempt was made to answer these questions.

With the subject seated in the usual posture a sphygmomanometer cuff was placed over the right upper arm and inflated to a pressure of 20 to 25 mm. of mercury, the left arm resting free in a similar position. After an interval of 15 minutes both forearms were injected with dye in the usual way and watched for 20 minutes, after which the pressure previously maintained on the right side was suddenly released and the progress of the dye observed for another 20 minutes. In these tests and in all to follow, when pressure was released the entire cuff was removed as rapidly as possible to avoid any obstruction whatever to lymphatic drainage. Text-fig. 3 shows the tracings from a trial typical of 5 that were made. But little of the dye entered the lymphatics of the obstructed side, as the tracings show, only a few small twigs filling upon injection. On the control side the usual network of capillaries appeared, and streamers began to form in the usual time. This might be regarded as a chance difference, since the findings after intradermal injections vary not inconsiderably from time to time and from individual to individual, were it not for the fact that it was noted quite regularly in every case in which the pressures employed were low, that is to say 15 to 40 mm. of mercury, and the venous circulation not severely hampered. On release of pressure streamer formation was so rapid that soon the dye distribution equalled that in the other arm.

In 10 other tests pressures of 30 to 40 mm. of mercury were employed for periods varying from 5 to 15 minutes. In these instances as in those just described, no lymphatic streamers were noted during the period of obstruction, but upon release dye streamers promptly appeared.



TEXT-FIG. 3. The effect of pressure on lymph flow in the arm. Tracings of the spread of intradermal dye in a normal forearm (left column) compared with that from a similar injection in the other forearm (right column). A pressure cuff inflated to a pressure of 20 to 25 mm. of mercury had been placed about the upper arm 15 minutes previously. The pressure was released 20 minutes after injecting this arm. The last three tracings in the right column show the effect of the release of this slight pressure after 3, 10 and 16 minutes respectively, as detailed in the text. $\frac{1}{2}$ natural size.

An abnormally rapid movement of lymph in the skin follows the release of lymphatic obstruction even when accompanied by only a slight impediment to the venous circulation. The findings, which were to have been expected, are preliminary to those which now

follow. They are an additional illustration of the fact that the results of dye injection serve as an indicator of even small differences in peripheral lymph flow, and that the failure of colored streamers to develop indicates the existence of lymphatic obstruction.

Still greater changes in pressure yielded significant differences.

In a series of 7 tests total venous obstruction, and hence lymphatic occlusion too, were effected for periods of 15 minutes to 1/2 hour, by throwing into the cuff pressures of 90 mm. of mercury. The arm became deeply suffused with blood. The lymphatic capillaries were far more easily injected than in the tests in which weaker pressures were used. (Compare the diagram in Text-fig. 3 with the photographs in Fig. 3.) The lymphatic capillaries were wider on the side where stasis existed and, during the injection, dye spread through them as far or even farther than in the normal arm, though it failed to proceed any further in the channels thereafter. While stasis was maintained no deep dye streamers appeared, indicating that there was no lymph flow.

Figs. 3 a to h show the results of an intradermal injection of 0.02 to 0.03 cc. of dye into a forearm 20 minutes after a pressure of 90 mm. of mercury had been thrown into a pressure cuff placed over the biceps. The photographs were taken 15, 30, 45 seconds, 1 minute and 2 seconds, 1 minute and 30 seconds, 2, 2½ and 3 minutes respectively after beginning the injection, which took 47 seconds. The first three photographs of the series, taken before much dye had escaped from the channels, show their actual size. Dye escape blurs their outlines in the later ones. 5 minutes after making the injection and 25 minutes after pressure was first begun, the cuff was released and removed as rapidly as possible. Immediately intense reactive hyperemia occurred during which a streamer began to form in less than a minute, with a suddenness and intensity of color never seen before. In all these tests the same phenomena occurred. The photograph, Fig. 3 i, which is typical, was taken but 2 minutes after the release of pressure, and it shows part of the deeply colored streamer formed in that brief period. This extended along the upper arm too, half way to the shoulder, and was longer and more brilliantly colored than the longest streamers arising from similar injections in normal arms even after half an hour. In most control tests such brilliant streamers are never seen at any time.

Peripheral Lymph Flow after Periods of Total Circulatory Obstruction

The findings suggest that some of the fluid accumulating in the tissues during and after complete venous or lymphatic obstruction, is rapidly removed by way of the lymphatics after the release of pressure. How much of the lymph flow results from this excess of fluid and how much is consequent upon reactive hyperemia cannot be said. The matter will be discussed below.

What are the effects on the lymphatics of complete cessation of the

circulation, either by sudden constriction of the limb, shutting off arteries and veins instantly, or by slow pressure occluding just the veins and somewhat later the arteries?

In 12 tests on 8 normal individuals, both types of obstruction were tried. In half, a sudden injection of air under 215 mm. pressure into the pressure cuff arranged on one upper arm induced total circulatory obstruction in a second or two, while in the other arm a period of 5 to 7 minutes of venous engorgement at 85 mm. pressure was allowed to elapse before total circulatory stoppage was brought about by raising the pressure to 215 mm. In the other half of the tests one arm was subjected to one of these procedures while the other, normal one served as a control. At varying periods after the occluding pressure had been put on small amounts of dye were injected in both arms. In some instances 4 or 5 injections were made in each arm with release of pressure immediately after the last injection. The period of total obstruction varied from 8 to 35 minutes, but the varying time interval did not appreciably affect the behavior of the dye.

In the majority of these tests, involving about 35 injections in 8 different subjects, the lymphatic capillaries seemed wider than usual and dye entered them quite as readily as in the normal skin. One did not find the resistance to injection observed in the instances in which partial venous and complete lymphatic obstruction were effected by lesser pressures in the Riva-Rocci cuff,—for example 25 to 35 mm. of mercury. However, many exceptions were noted, and one cannot state that the lymphatics of the skin are always dilated in brief periods of total circulatory obstruction of the limb.

With the release of pressure reactive hyperemia developed at once, and with it, in both types of test, an extraordinarily rapid streamer formation indicative of very active lymph formation and flow. In a few seconds deeply colored streamers suddenly appeared 15 or 20 cm. in length and of an intensity never equalled by such small injections of dye in any of the tests so far described. Even pale streamers of this length require approximately half an hour or more to form under normal circumstances. A brief description of a typical test follows.

As the subject sat with both arms resting on a table before him, a pressure of 215 mm. of mercury was abruptly thrown into the cuff on one upper arm, and maintained for 16 minutes. At intervals of approximately 3 minutes thereafter 4 dye injections were made in the now livid skin of the volar surface of the arm. The spread of the dye at each situation was photographed and traced and observations were also made under the microscope. In these tests it was planned to release pressure in less than a minute after making the last injection, while dye still remained within the lymphatic capillaries and could be observed under the

binocular microscope. 29 minutes after first obstructing the circulation, pressure was suddenly released while the fourth colored area, injected but a minute before, was carefully watched through the microscope. There was no sudden movement of dye within the superficial lymphatic capillaries. After a few seconds reactive hyperemia appeared, and in less than 40 seconds dye was carried up the arm in the subcutaneous lymphatics for a distance of 15 cm. These streamers, 1½ and 3 minutes respectively after the release of pressure, appear in Figs. 4 *a* and *b*. They well show the intensity of the color.

The upper left arm of the same subject was now bound with the pressure cuff and the test repeated save that pressure was first raised to 85 mm. of mercury for 6 minutes to occlude the lymphatics and engorge the arm. At the end of this period pressure was abruptly raised to 215 mm. and at the same intervals as in the preceding test dye was injected. The reactive hyperemia following release of pressure brought about no sudden movement of dye in the lymphatic capillaries, which could be made out under the microscope, but as before several streamers appeared with great speed and became intensely colored. Such streamers, appearing after periods of total circulatory obstruction preceded by venous occlusion, are shown in Figs. 7 and 8. Their significance will be discussed below.

In one trial, upon another subject receiving but a single injection, dye passed in less than a minute into 2 broad lymphatic channels and there developed what resembled beginning streamers but these progressed no further during the remaining 15 minutes of circulatory obstruction. Extensions of dye such as these were observed but rarely and only in arms which had been subjected to total circulatory obstruction for 15 or 20 minutes. When they appeared they extended, not upwards as did the genuine streamers, but transversely across the arm in the direction of the floor as if the dye were moving by gravity in lymphatics already distended by stagnant fluid. 2 minutes after the release of pressure, a long streamer appeared, running from the tip of the dye extensions to the elbow. In this particular instance, the injected area and the region about the extensions of dye whealed at the same time. The skin containing the streamer which had formed during this period also whealed a minute later. A wide hyperemic flare was present about both the streamer and the injected area. Fig. 5, a photograph taken without color screening, 4 minutes after release of the pressure, shows the flare extending up the arm about the dye-containing lymphatic. As can be seen in the photograph, the streamer takes its origin from the 2 laterally directed dye extensions described above.

The findings disclose a very rapid formation and flow of lymph following periods of total circulatory obstruction, equally rapid whether or not the limb has been previously engorged with blood. Reactive hyperemia in an arm deprived of oxygen seems to have been responsible for the results, for no large amount of fluid can have accumulated outside the vessels of the arms in which the circulatory

arrest had been abrupt. Undoubtedly the blood vessels were more permeable, after the period of circulatory arrest, as Lazarus-Barlow (20) and Landis (15) have shown, and, with the advent of reactive hyperemia, they furnished much fluid for lymph.

Retrograde Lymph Flow.—Following the release of pressure in 2 of the 8 subjects and during the subsequent reactive flush, a single streamer of dye appeared, running down the arm toward the wrist for 7 cm. in one instance and 15 in the other. In the latter instance a streamer ran proximally as well, both streamers appearing in a photograph, Fig. 6, taken 2 minutes after the release of pressure. Retrograde flow of dye has never been found save after total circulatory obstruction or in severe cardiac edema, as described in the following paper.

Reactions of Lymphatic Capillaries within Bier's Ischemic Patches

When an arm is engorged by venous obstruction and shortly thereafter all circulation is stopped for several minutes, irregular pale areas,—the ischemic patches of Bier (21),—appear in the skin, interspersed among purple congested regions. The engorged cutaneous vessels contract in local areas and drive blood into adjacent regions which in turn become congested. It seemed a matter of interest to determine whether the activity of the blood vessels responsible for the blanching in Bier's spots is accompanied by corresponding changes in the lymphatics. Intradermal injections of minute amounts of dye were accordingly made into Bier's spots and the flushed areas surrounding them.

In human skin Bier's spots are best elicited by total obstruction of the blood flow in a limb that has previously been overfilled with blood by venous occlusion. We determined the blood pressure of the 8 volunteers employed in the work just described, and after the usual rest period the subject was placed at the low table with the forearm, volar surface up, and propped only at the wrist and elbow. It has been shown by Rous and Gilding (22, 23) that Bier's spots are prone to appear on the upper side of a horizontally propped arm supported in this way. A pressure of 70 to 80 mm. of mercury thrown into a sphygmomanometer cuff adjusted about the upper arm, maintained venous occlusion for 6 or 7 minutes. At the end of this period the pressure was abruptly raised to 200 mm. of mercury to secure complete stasis. When the pale and purple mottling of the Bier's spots and congested areas was well advanced, an intradermal injection of dye solution

was made into the largest Bier's spot available. As usual the progress of dye was watched through the binocular microscope to distinguish the ease or difficulty with which it entered the lymphatics, and to observe their state of dilatation or contraction, the rate of dye escape, the extent to which dye moved within the channels and any evidences of lymphatic drainage. Photographs were taken as usual. 5 to 8 minutes later a similar injection was placed in a highly congested purple area of skin and the fate of the dye compared with that of the first injection. During this period occasional photographs and observations were made of the region first injected. Finally a third injection was made into another Bier's spot. As soon as dye entered the lymphatic channels and before its escape had occurred the pressure was released and the cuff removed as usual. Under the binocular the effect upon the dye movement in the lymphatics was sought while other observers with hand lenses watched the two regions first injected.

For such tests a period of total occlusion of 40 to 45 minutes sufficed. At once upon release of the pressure brilliant reactive hyperemia occurred, with immediate and striking formation of streamers. In alternate instances the order of the injections was reversed, that is to say the first injection was made into a congested area, the second into a Bier's spot, but the last always into another Bier's spot.

It is well known that injury to the skin leads to vasodilatation (24) and it was feared that the prick of the injecting needle might of itself destroy the contraction of blood vessels within Bier's spots. In our work it seemed wise to test the point in each of the 8 subjects before injecting dye. In scores of Bier's spots insertion of the hypodermic needle without expulsion of dye or else with the injection of 0.02 cc. to 0.03 cc. of Tyrode solution has failed to change the blanched appearance. When dye was to be injected, spots previously untouched were employed, of course.

The very marked contrast in the caliber of the blood vessels within Bier's spots and the surrounding congested areas is not duplicated in the lymphatics. Nonetheless there are noticeable differences.

Within the Bier's spots we have found the caliber of the lymphatic capillaries to be variable, much constricted in 4 of 8 individuals and approximately as in normal skin in the others. They were usually definitely narrower than in the congested areas of the same arm but in 2 of the 8 instances the difference was not definite. Less dye appeared to enter the lymphatic capillaries within Bier's spots, that is to say fewer channels became visible than in normal skin, or in the congested areas of the test arm; and during and after the injection the dye passed but a little way from the point of the needle. As result the interstitial bleb was relatively large, the amount of dye within the lymphatics small. Dye escaped from the injected channels within Bier's spots more slowly than from those within the dark congested regions and even more slowly than in normal skin. Secondary diffusion of escaped dye was practically absent.

In the congested skin the lymphatic capillaries were usually dilated as one

would expect from our earlier tests with venous obstruction. In 2 of 8 instances they were found about normal in appearance. Always in these regions dye passed readily from the needle into the lymphatics, the amount of fluid remaining interstitial being small, that within the lymphatics large. Dye was carried farther in the lymphatics of a congested area than in a Bier's spot or in normal skin. As a consequence the area of coloration seemed greater for a given amount of dye. The colored fluid escaped far more rapidly from the lymphatic capillaries in these areas and occasionally one could see slight movement of dye into a drainage trunk for a centimeter or more, as in the previous experiments involving total circulatory obstruction.

In Fig. 7, photographs *a* to *e* were taken at 15, 30, 45, 90 and 107 seconds respectively after beginning the injection into one of the congested areas of the arm of a healthy young man, with blood pressure 118/80. The injection required but 43 seconds. 4 minutes later photographs *a'* to *e'* were taken at similar time intervals during and after a similar injection into a Bier's spot. Owing to the red color filter used the spot cannot be seen. One can note however that the injection into it required more time, 59 seconds, for the needle is seen in one more picture; the spread of dye in the lymphatics is less and dye escape from them much slower. Photographs *f* and *g* will be discussed below. The result is typical of the majority of the experiments.

The apparent resistance to the entrance of injected dye offered by the lymphatic capillaries within Bier's spots is probably due to the narrowing of the channels, for this showed itself in a different way in a test now to be described.

The injecting needle had been inserted in the center of a small Bier's spot and by the 15th second after beginning the injection (Fig. 8 *a'*) the dye had entered scarcely any channels. As pressure was continued (Fig. 8 *b'*) colored fluid suddenly darted into 2 or 3 channels and, in them, passed completely through the blanched area to congested skin beyond. Once in the latter it swept around the pale area, still in the purple skin (Fig. 8 *c'*). The circumference of the Bier's spot, not visible in the photograph because of the screening, lay just within the broken ring formed by dye in the lymphatics. There was no further filling of channels within the Bier's spot itself, and as result there formed only the interstitial bleb shown in the pictures, with later some secondary diffusion of dye therefrom. The injection in the congested area required about 44 seconds, that in the Bier's spot 61. Secondary dye escape from the circumferential channel, lying within the congested skin, was quite marked (Figs. 8 *c'* and *d'*), as also was that within the Bier's spot itself. The photographs were taken at intervals identical with those of Fig. 7, as shown by the legends.

In all these tests no significant widening or constriction of the lymphatic capillaries took place immediately upon release of the pressure, either in the Bier's

spots or in the congested areas. Nor was there any sudden visible extension of dye in the superficial channels, or movement of that which was interstitially placed. With the development of the usual violent reactive hyperemia, intensely colored streamers of dye extended from each of the injection sites even more suddenly and with greater intensity than in the preceding experiments. Within 15 to 20 seconds after release of pressure, in 6 of the trials upon 4 of the 8 subjects, colored streamers of great intensity extended 16 to 20 cm. above the site of injection. In the remainder similar ones invariably developed within 1½ or 2 minutes. Such streamers were always far more intensely colored than those arising from injections in normal skin, even after half an hour or more.

For example in Figs. 7 *f* and *g* the arm is shown 1½ and 3 minutes respectively after the release of pressure. The injected area, shown in detail in the series of photographs *a* to *e*, lies in Figs. *f* and *g* directly above another injected area which is the one shown in photographs *a'* to *e'*. The 3rd injection of the test was made into a Bier's spot immediately before releasing pressure, and consequently no detail photographs were taken of the dye spread. This appears nearest the wrist in photographs *f* and *g*. Figs. 8 *f* and *g* show the formation of dye streamers at similar intervals after release of pressure, as they occurred in the second test. The 2 dark spots from which no streamers appear are dots of dye placed upon the surface of the skin and hence have no significance.

Reverse Flow of Lymph in Certain Instances.—Following the release of pressure, in three of these instances, retrograde colored streamers moved toward the wrist in the subcutaneous lymphatics, as in some of the tests described above in which complete circulatory obstruction had also been induced. Such a streamer is shown in Figs. 8 *f* and *g*, running toward the wrist from the lower injected area. It is well known that the valves of the deeper layer of lymphatics normally resist retrograde flow.

DISCUSSION

In the present work changes in the character of the peripheral lymph flow have been demonstrated by the visible passage of small amounts of intradermally injected dye from the superficial lymphatics to subcutaneous channels in which, as it extended subsequently up the limb, it gave the appearance of colored streamers.

The dye of the streamers was in the lymphatics. This has been shown repeatedly in animal experiments in which after the injection of small quantities of dye into the skin of the lower portions of limbs or ears the pigment has been found in the subcutaneous lymphatics, diluted and pale and draining to the nearest lymph node. Can one

assume that the extension of the colored streamers in the present work is truly indicative of a flow of lymph? The streamers were always short and pale at first. They gradually became longer in 15 or 20 minutes, still pale at the upper end but darker near the site of injection as if the lymph flowing in the subcutaneous channels had been stained by gradually increasing amounts of dye coming to it from the site of injection. The fact should be stressed that this is a very different phenomenon from that obtained when larger amounts of dye are forcibly injected into skin to obtain anatomical preparations of the lymphatics. In such injections, undiluted dye is actually forced into the subcutaneous channels, not transported by the flowing lymph. In the present work the dye solution was injected with as little pressure as possible. This pressure seems to have been of little importance, for when similar amounts of fluid dye were deliberately introduced with great force no streamers appeared until after the usual time. Evidently the small amounts of fluid (0.01 cc. to 0.04 cc.) which were injected, largely into the interstitial tissue and only partly into the lymphatics, were not enough to account for the formation of long streamers of color. Further, great differences in streamer length and intensity occurred corresponding to known changes in lymph flow, although the amount of dye fluid and the pressures at which it was injected remained constant. Attention has already been called to many such instances. For example, in normal, horizontally placed limbs the streamers required some minutes to develop, but during the reactive hyperemia following release of obstruction of the circulation they appeared immediately. Streamers were found short and faint in the resting limb but long and intensely colored in the warmed but also resting limb. Had the streamers been due to pressure alone they would have been approximately uniform in all instances. It is plain that intradermal injections of dye demonstrate changes in the flow of peripheral lymph and that the method can be used clinically.

The superficial lymphatics appear to be about the same in width from day to day in the normal skin of any one individual. Variations in the width of the lymphatic capillaries can be recognized, even though the changes are not great. For example, in the ischemic skin of Bier's spots and in the adjacent, congested areas they are

narrow and wide respectively, but the differences are not as marked as those shown by the blood vessels. Following other changes in the physiological state of the skin the lymphatic capillaries have appeared wider or more narrow, but much stress should not be laid upon these differences save when they are pronounced. For the method is qualitative at best and some individuals vary considerably from the average in the width of their lymphatics. Whether the variations in caliber are passive or are the result of active contraction we are unable to say.

The tests have yielded direct indications of what happens within the skin lymphatics. In the resting limb horizontally placed there is slight continuous lymph formation and flow. Warmth with its accompanying hyperemia produces longer and more brilliant streamers than those appearing in normal skin. Lymph flow is well known to be increased during the hyperemia of heat (4-6). When the arterioles are dilated, capillary pressure is increased, as Landis (15, 25) and others (26-28) have shown, fluid escape from the blood vessels is greater and dyes injected into the blood stream pass more rapidly into the tissues (29). Muscular movement, massage,—even passive movement to a mild degree,—increase lymph flow as already known and as the present experiments attest anew.

The effects of posture are striking. In the arm intradermally injected with dye and held vertically downward at rest, lymph flow is absent, as judged by the lack of streamer formation. When the same arm is raised vertically above the head and then injected, lymph flow is found to be rapid. It is absent in the injected lower legs of normal subjects seated quietly with the feet resting on the floor, but becomes conspicuous in the form of long and highly colored streamers when the injected leg is propped on a table or desk while the subject remains seated. As is well known capillary pressure is high in dependent portions of the body (15). Standing diminishes blood volume (17-19) and the legs increase in size (17). Landis and Gibbon (16) found fluid accumulation in the arm at the rate of 0.2 cc. per minute per 100 cc. of tissue when venous pressure was increased to 80 cm. of water. In an arm or leg hanging vertically downward and at rest, the effect of posture is practically that of an increased lymphatic and venous pressure. The mechanism is charged as it

were for increased lymph formation. But the present experiments prove that there is no lymph flow in the cutaneous lymphatics of the intact, motionless, dependently hanging arm or leg,—no activity on the part of the lymphatic system to relieve its increasing fluid content even though the lymphatics themselves may fill and become distended, as will be demonstrated in the following paper. With muscular activity, though, lymph is forced up the leg against gravity. Changes in the position of the limb in relation to the body, friction, rubbing and intermittent changes in tissue pressure brought about by bodily activity are of great importance for lymph flow, certainly greater under some circumstances than are the physicochemical changes occurring in the limb.

Lymph flow was observed to cease in the subcutaneous channels of the forearm when, by means of a cuff about the upper arm an external pressure was applied which was far less than that required to obstruct venous flow. Upon release of the pressure the lymph flow was greater than in normal skin. The fluid which may have accumulated in the tissues during the period of pressure was apparently removed in part by way of the lymphatics. Lymph flow was still more rapid after the release of greater pressures, sufficient to occlude the veins and engorge the arm with blood. The most active lymph flow we have ever observed under normal or pathological conditions, as judged by streamer formation, has occurred during the reactive hyperemia following upon the relief of total circulatory obstruction. It was equally great whether or not the arm had previously been engorged with blood as described above. Lazarus-Barlow (20) has shown that total occlusion of blood flow leads to little transudation into the tissues but that lymph under these circumstances is rich in protein, presumably the result of increased capillary permeability (15). No doubt the intense lymph flow observed in our experiments during the reactive hyperemia following total circulatory obstruction is to be attributed to a greatly increased circulation through a vascular bed which had previously been deprived of oxygen and had become more permeable than normal (15). The same assumption will explain the streamer formation which arose from injected ischemic Bier's spots and from their neighboring congested areas, following the release of circulatory arrest. In the congested areas local accumu-

lations of fluid must have occurred; in the ischemic areas, no doubt, they were less; but from both streamer formation was equally great. The intense reactive hyperemia coming on at once after release of pressure obliterated the local effects. These experiments show further that, under certain conditions, lymph flow can be great in a resting limb.

Tests to demonstrate the influence of cold upon lymph flow were attempted but the occurrence of hyperemia rendered the results indecisive. Cold lowers capillary pressure for a few minutes (15, 25, 27) but after a variable time reactive hyperemia occurs (25). One would expect lymph flow to be oppositely affected by the two conditions but the dye streamers were not sufficiently different from those appearing in normal skin to warrant any conclusions. Unfortunately too the methods employed did not allow us to demonstrate the effect of rest upon a previously active limb, or of cold upon previously heated skin. Dye draining in subcutaneous lymphatics escapes to some extent into the surrounding tissues. A slight difference in the length or intensity of a colored streamer cannot be appreciated because of the escaped dye. Clearly, however, the dye method brought to light the existence of less lymph flow in the control limbs.

One further point deserves mention. When dye is injected intradermally and the skin immediately sucked, much of the color is driven into the lymphatics draining the injected area.. Once within these channels it cannot be recovered by suction but instead is driven farther along in them. By sucking a wound or a cut much material may be removed and its subsequent entrance into blood vessels or lymphatics prevented; but much is also distributed widely in the lymphatics.

SUMMARY

Vital dyes injected intradermally enter lymphatic capillaries directly, rendering them visible, and appear later in the draining lymphatic trunks as colored streamers. The method enables one to perceive the state of the lymphatic channels and the rate of lymph flow within them. It yields consistent results when tested under physiological conditions known to increase or decrease lymph flow.

In the horizontally placed normal limb at rest there is slight lymph flow. In a normal leg or arm hanging downward lymph flow ceases although fluid in the limb increases. When a previously dependent arm is raised above the head, or when the foot of a seated subject

is propped on a table, lymph flow in the raised limb becomes active. It ceases in the skin of an arm subjected to partial obstruction of the veins by pressure from without, but very active lymph flow appears during the reactive hyperemia which follows upon the release of venous obstruction. It is still greater following release of total circulatory obstruction, and seems to be the same whether or not the limb has previously been engorged with blood. In the ischemic patches which appear in the skin of a limb during total circulatory obstruction (Bier's spots) the lymphatic capillaries are definitely and considerably constricted, whereas they are slightly dilated in the purple, congested regions of the skin round about. On release of obstruction there occurs a strikingly rapid, equal lymphatic drainage from both regions.

The significance of all the findings is discussed.

When dye is injected intradermally and the skin sucked, much of the foreign material is driven into the lymphatics draining the injected area.

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EXPLANATION OF PLATES

PLATE 11

FIGS. 1 and 2. Natural size photographs of the distribution of dye on intradermal injection into the skin of the volar surface of the normal arm. The photographs were taken at intervals of 15, 30, 45 seconds; 1, 1½, 2, 3, 4 and 20 minutes after beginning the injections which lasted 52 and 48 seconds respectively. Fig. *i* is reduced to 1/7 natural size.

Note the size of the interstitial bleb of dye at the needle point in proportion to the amount within the lymphatic capillaries. Dye escape soon caused blurring of their outlines. The last photograph of each figure taken after 20 minutes at a distance of 40 cm. shows a pale blue streamer extending up the arm from the injected area.

PLATE 12

FIG. 3. Results of an iotradermal injection of dye in the skin of the volar surface of an arm during a period of venous obstruction caused by inflation of a Riva-Rocci cuff to a pressure of 90 mm. of mercury. The injection, which required 47 seconds, was made after pressure had endured for 20 minutes. The natural size photographs *a* to *h* were taken at intervals of 15, 30, 45 seconds, 1, 1½, 2, 2½ and 3 minutes respectively after beginning the injection. Fig. *i* is reduced to 1/7 natural size.

The lymphatic capillaries are slightly dilated. The final photograph shows only part of an intense blue streamer which formed in the 2 minute period after release of the pressure and extended along the upper arm half way to the shoulder. Prior to release of the pressure no streamer existed.

FIG. 4. Streamers developing in 1½ and 3 minutes following the release of total circulatory obstruction as described in the text. Their intensity should be noted. $\times 1/7$.

FIG. 5. Skin injected with dye during a period of total circulatory obstruction occasionally whealed upon release of pressure, and showed an hyperemic flare about both the injection and the resulting streamers. Total circulatory obstruction of an arm was maintained for 15 minutes, and then the usual dye injection

was made in less than 1 minute. No streamers developed during another 15 minute period of obstruction. 2 minutes after release of pressure a streamer formed extending almost to the axilla. At this time the injected area, and a minute later the skin adjacent to the streamer whealed. The photograph was taken without a color screen at the 4th minute after the release of pressure. It shows the extensive hyperemic flare about the injection and accompanying the streamer as far as the elbow. $\times 1/2$.

FIG. 6. The appearance of retrograde streamers. The usual intradermal injection of dye was placed in the forearm 26 minutes after total circulatory obstruction was effected. No streamers developed during the period of obstruction. The photograph shows 2 streamers as they appeared 2 minutes after release of the pressure. One of them is a retrograde streamer extending to the wrist. $\times 1/7$.

PLATE 13

FIG. 7. A comparison of the characteristics of the lymphatic capillaries in deeply congested skin (*a* to *c*) surrounding a Bier's spot, and within the Bier's spot itself (*a'* to *c'*). The photographs were taken at the same time intervals after dye injection (see text). Figs. *f* and *g* show that streamers of equal length and intensity had developed $1\frac{1}{2}$ and 3 minutes after release of the total circulatory occlusion. This had endured for about 40 minutes and had produced the Bier's spots. The injected areas shown in the detail photographs (*a* to *c* and *a'* to *c'*) lie in a nearly vertical line in Figs. *f* and *g*. The lower is where the Bier's spot was. The third spot shows the injection which was made into another Bier's spot just before release of pressure, and no detail photographs were taken. A streamer is beginning to form from it. The detailed photographs are natural size. Figs. *f* and *g* are reduced to $1/7$ natural size.

FIG. 8. Photographs taken in a test similar to that which furnished Fig. 7. They are described in detail in the text. Magnifications as in Fig. 7.

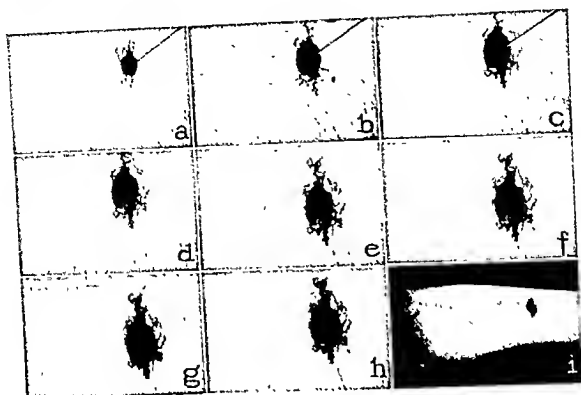


FIG. 1

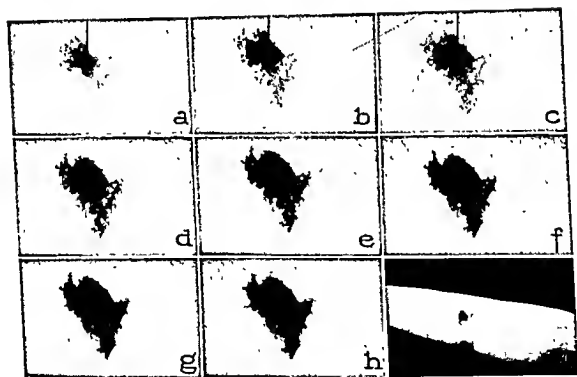


FIG. 2

(McMaster: Cutaneous lymphatics and lymph flow)

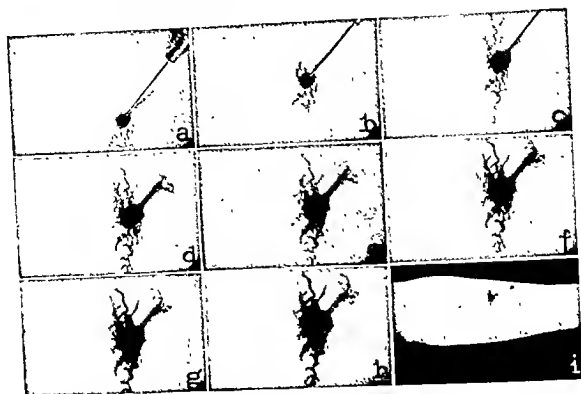


FIG. 3



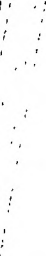
FIG. 4

FIG. 6



FIG. 5

(McMaster: Cutaneous lymphatics and lymph flow)



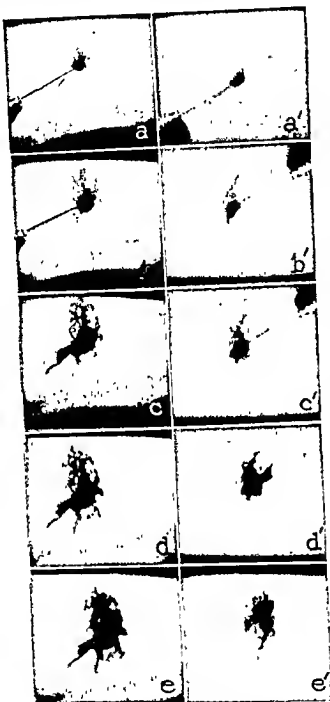


FIG. 7

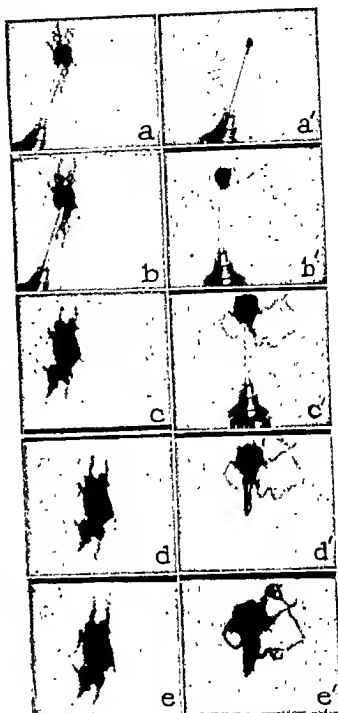


FIG. 8

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THE LYMPHATICS AND LYMPH FLOW IN THE EDEMATOUS SKIN OF HUMAN BEINGS WITH CARDIAC AND RENAL DISEASE

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PLATES 14 to 16

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Lymph flow in human skin, even in a resting limb, is far more rapid than has been generally supposed (1, 2). The fact has led us to investigate the share taken by the lymphatics in the removal of fluid from the skin of patients suffering from the edema of cardiac and renal disorder. It seemed reasonable to assume that lymph flow in patients with long standing edema from circulatory failure might differ radically from that of individuals afflicted with renal disorder and edema but without any circulatory failure. For the purposes of the present study patients of the two classes were selected. They will be referred to as cardiac or nephritic respectively. Those of the latter sort were all individuals under 30 years of age, with acute or chronic hemorrhagic nephritis. All had marked or moderate hypoproteinemia, and at some time during our studies edema and pronounced albuminuria. No nephritic patients were included who showed any evidence of cardiac decompensation. The patients with cardiac edema on the other hand were mostly over 60 years of age, and had suffered repeatedly from cardiac incompetence with edema of the lower limbs and other classical symptoms of circulatory failure. Patients yielding any signs of renal disease were excluded from the group. We are well aware that some renal disorder must inevitably follow in the train of cardiac disease, but it was too slight to merit consideration in the patients selected for this study.

Method

The technique described in an accompanying paper (1) was utilized to demonstrate changes in the lymphatics and in the lymph flow.

The method depends upon the intradermal injection of a vital dye, patent blue V, which renders the lymphatic capillaries visible (1-5). The sequence of events following such an injection in normal skin has already been recorded (2). The injected dye enters the rich cutaneous network of lymphatics and spreading through them appears as a highly colored net; but some remains as a bleb in the interstitial tissue. After the removal of the injecting needle the dye continues to spread through the minute lymphatics, increasing the area in which they are visible. Diffusion from the channels takes place rapidly. Within a few minutes it is noticeable that one or several extensions of dye have taken place, for a coloration of some of the larger draining lymphatics attests to the movement of fluid along them. When this occurs these deeper channels appear like colored streamers through the skin.

To render the findings comparable with those presented in the previous paper (1) the same dye solution was used and in the same concentration, the amounts varying from 0.02 to 0.04 cc. as the experiments required. The smallest volume was employed whenever a patient was injected for the first time. In nearly all instances larger amounts of dye were given in subsequent tests.

In addition to the elderly patients above mentioned, with cardiac disease and various stages of edema, and the young patients with nephritis, a few persons living under the same hospital conditions and suffering from diseases not characterized by edema or circulatory disturbance were utilized. Normal individuals, young and old, served as controls. During the observations and for half an hour prior to the first dye injection, the normal subjects were kept at rest in a position similar to that of the patients. Save where especially mentioned the patients had been confined to their beds and during the observations they remained semi-recumbent, with both legs horizontal. They were usually injected intradermally on the anterior surface of the ankle at the level of the malleoli of the tibia, where edema is usually intense, but the injection site was shifted a little when this was necessary to avoid varicosities, dilated venules or other skin lesions. When possible, tests were repeatedly made upon the same patients in different stages of the disorders studied—during the formation of edema, while it remained stationary and during its disappearance, as will be described below.

Changes in lymph flow during the increase or decrease of transient edema were studied in ambulatory patients who acquired the edema upon standing and lost it when reclining. These patients were injected in one ankle and observed for half an hour while the legs remained horizontal. They were then allowed to stand or sit, with the feet down, and injected again as the edema appeared. When obvious edema had developed they were put to bed and 10 minutes later were injected while the edematous leg rested in a horizontal position.

The tests were made as in the preceding paper, the binocular microscope, camera and lighting device extending over the bed on an adjustable metal stand. Thus there was no need for the patients to move. At intervals for 3 to 5 hours after the injections the sites were examined to observe the colored streamers and the interstitial spread of the dye bleb.

Findings under Normal Conditions

It is necessary to consider first the state of affairs following the introduction of dye into the skin of the ankle of normal individuals. Scores of tests on 10 normal volunteers have shown that injections of 0.02 to 0.04 cc. of patent blue V yield information on the rate of lymph flow in the skin of the ankle and leg. Colored streamers develop in the deeper lymphatics which are long or short, pale or deep colored, under conditions which are known to increase or decrease lymph flow respectively. Some of the observations have been described in the preceding paper.

Figs. 1 *a* to *i* show the typical findings after dye injection into the skin of the ankle of a normal individual with the leg horizontal and at rest for 3/4 hour. The pictures were taken 10, 16, 25, 40 seconds, 1 minute and 1 second, 1 minute and 50 seconds, 2 minutes 45 seconds, 3½ minutes and 20 minutes, respectively, after the beginning of an injection which extended over a period of 62 seconds. All but the last picture are reproduced in natural size, and they show the bleb of dye at the point of the needle, the extent to which dye entered the lymphatic capillaries and the later spread within them. Dye escape from the channels appears as a fuzzing or blurring of their borders (Figs. 1 *f*, *g* and *h*). The last photograph, taken 20 minutes after the injection, shows 2 small streamers which extended up the leg for a distance of about 10 cm. In this connection it should be noted that streamer formation is less in the normal skin of the leg than in that of the arm, and the lymphatic capillaries are narrower.

Findings in the Edematous Skin of Cardiac Disease

More than 100 tests in 20 individuals with cardiac disease and edema of the lower limbs have all yielded evidence of stagnation of lymph, and incompetence of the lymphatics.

The patients selected for this portion of our study varied in age from 52 to 67 years and were chosen, as already stated, because they showed marked cardiac incompetence with little or no evidence of renal disorder. Tests were usually made shortly after their entrance into the hospital, when the edema was still increasing in the lower legs. Later they were injected while the edema seemed stationary and again while rapidly losing fluid as result of rest or therapeutic measures. The tests were repeated when the patients became edema-free in the

ankles as indicated by the lack of pitting on pressure, and in a few instances were repeated 2 or 3 weeks later while edema was still absent. When possible several tests were carried out in each of these stages.

The results can best be presented by a brief consideration of some typical findings obtained in a single individual in the various stages of edema formation and resolution.

The patient, 63 years of age, had experienced repeated cardiac incompetence in the previous 6 years. For several days before entering the hospital he had been confined to bed. Both legs were congested and dark red, showing many dilated venules and bluish purple varices. In 37 seconds 0.03 to 0.04 cc. of dye solution was injected. It entered the lymphatic capillaries far more readily than in normal persons with result that there remained at the point of the needle only the smallest bleb of interstitial dye. The channels were very wide, and during the injection dye passed much farther along them than in the lymphatic capillaries of normal skin. This is shown in Figs. 2 *a*, *b* and *c*, which, like Figs. 1 *a*, *b* and *c*, of a normal subject, were taken 10, 15 and 25 seconds after beginning the injection. The colored area extended beyond the limits of the photograph of Fig. 2 *c*, taken after 25 seconds, and 20 seconds later the spread of color had become so great that the position of the camera was shifted and four photographs taken at short intervals to include all the region of the dye-containing lymphatic capillaries. The shifting was done during the period from 3/4 of a minute to 1 minute 15 seconds after the beginning of the injection. The overlapping areas of the four photographs were cut away and the remainders pieced together to furnish Fig. 2 *d*. Such shifting of the camera was never necessary in tests upon normal individuals.

The photographs show the dilatation of the superficial lymphatics and the plexus richly injected, demonstrating the presence of free intercommunication between the channels even better than in normal skin. Secondary dye escape was more rapid than in normal skin. Furthermore, dye appeared in many channels at a greater or less distance from the stained area, like little disconnected twigs of color. The phenomenon, mentioned here in passing, will receive consideration further on. In Fig. 2 *f*, the arm and the stained region are shown after 20 minutes. The lymphatic capillaries were now colored over a wide area, yet the same amount of dye had been used as in the tests yielding the photographs of Fig. 1. No colored streamers appeared at any time in the deep draining channels. In other words there was no evidence of lymph flow.

A month later the patient's edema began to decrease rapidly. On the 3rd and 5th days of active diuresis, while fluid loss continued but edema was still present, intradermal dye tests were made again in both legs. The findings in the first of these tests are shown by photographs *a* to *f* in Fig. 3, taken at approximately the same intervals as some of those in Figs. 1 and 2, that is to say at 18, 25, 35 seconds, 55 seconds, 2 minutes 20 seconds, and 20 minutes, respectively, after beginning the injection which lasted 50 seconds. The findings were similar to those just de-

scribed. Again there were no streamers of dye, which would have evidenced lymph flow. From the local spread of dye one could not tell whether edema was increasing or decreasing.

A week later, 3 days after the patient had become edema-free, as judged by the absence of pitting on pressure, the tests were repeated. The lymphatic capillaries in both legs appeared just as before, and an exceedingly wide area was colored by the dye. In other respects too the results were similar to the previous ones, save perhaps that dye escaped less rapidly from the channels. Despite the subsidence of the edema the lymphatic capillaries had remained dilated, and no streamers developed to indicate flow in the deeper lymphatics.

In many instances of long standing cardiac edema isolated "islands" of dye-containing superficial lymphatics appeared during the actual course of the intradermal injections, frequently 10 to 12 cm. away from the point of the injecting needle, though more often closer. They were separated from the area of staining by skin of normal hue. These islands were never seen under normal circumstances. It was plain that some of the dye, injected into the superficial plexus of lymphatics, had passed into those of the deeper layer, and spread along them unseen to emerge again at some distance, in the superficial plexus.

The six photographs of Fig. 4 show a typical finding of this sort. They were taken at intervals of 20, 30, 55 seconds, $1\frac{1}{2}$, $3\frac{1}{2}$ and 20 minutes, respectively, after beginning the injection, which required 48 seconds. Photograph *b* was taken about 10 seconds before the first pair of islands appeared, which are seen in *c*. To obtain Fig. 4 *d* the camera was moved slightly to show 2 more islands which had appeared in the interval. This picture was taken $1\frac{1}{2}$ minutes after beginning the injection. The next figure, 4 *e*, shows the rapid escape of dye from these islands that occurred in a further period of 2 minutes. At the time of the injection itself, or just a few seconds later, several other islands appeared, too far away to be photographed. These are shown in Fig. 4 *f* as they appeared 20 minutes after the injection. One group, indicated by the arrow at A, appeared 8 cm. away from the original injection site, below and far back at the heel, the distance being diminished by perspective. The other group, shown by the arrow B, was situated 3.5 cm. above the immediate area of injection. The islands of dye appearing in Figs. 4 *a* to *c* are easily distinguished in Fig. 4 *f*. The dark shadows running up the leg above the arrows A and B are merely groups of dilated venules.

Islands such as are here described often appeared below, that is to say distal, to the site of injection in cardiac patients, as well as toward the trunk or at the sides. A valvular incompetence of the deeper lymphatics, due to widening of them, will explain them all.

Fig. 5 shows the phenomena to better advantage. The injection was made into the ankle of a patient with marked edema which was rapidly decreasing through profuse diuresis. The photograph was taken half an hour after beginning the injection, which required less than a minute. The arrow A shows the point at which the needle entered. Dye escape from the channels and its interstitial spread has obscured the lymphatics but several more or less confluent islands of color can be made out. A semilunar strip of more lightly colored lymphatics indicated by the arrow B was originally completely separated from the main coloration. Above this, as seen in the photograph, there remains a small isolated patch of color. The large continent of dye indicated by arrow C was originally made up of fine islands now joined owing to diffusion of dye. The figure is shown at this stage to illustrate the fact that even half an hour after an injection in a cardiac patient with marked edema that was rapidly disappearing no evidence was obtained of lymphatic drainage. At the upper part of the stained area some deep lymphatic channels are seen containing colored fluid, but these had filled during the injection, and the dye did not pass further along them.

The findings in our other tests bear out those just described. When dye was injected into the skin of the ankle of a patient with cardiac edema the material entered the superficial lymphatics much more readily than in normal skin. The lymphatic capillaries were seen to be widely distended and apparently filled with fluid, for at the periphery of the injected regions there often appeared an abrupt paling of dye within the distended lymphatic capillaries themselves, as if from dilution with a copious fluid already present in the vessels. Almost all of the injected dye entered the lymphatics directly, the interstitial bleb at the point of injection being negligible. As result dye extended much farther and colored a wider area than in the normal individual, the intercommunications between the channels being, to all appearance, freely open and the injection of the superficial network in consequence very complete. Furthermore dye escaped from the channels with far greater rapidity than from those in normal skin. In patients with slight or moderate edema the changes were less marked. The studies as a whole disclosed every gradation from the normal to a state even more changed than that described, depending upon the condition of the patients.

We have never observed the formation of colored streamers in the edematous skin of horizontally resting legs of patients with cardiac edema, despite the distention of the lymphatic channels. In other words there is none of the evidence of lymph flow seen in the normal

limb. Dye merely diffuses from the lymphatic capillaries into the tissues and colors a widespread area which loses its blue hue gradually within the next 24 hours. The failure of streamers to appear is not due simply to the passage of the dye into great reservoirs of tissue fluid in which it is retained or dispersed. In the skin of patients with cardiac disease almost all the dye entered the lymphatic capillaries directly and, as the photographs show, remained within them long enough to indicate movement of the lymph had it occurred. (Compare Figs. 2 and 3 with Fig. 6, of the edematous skin of a nephritic individual.) After a few minutes most of the dye had escaped into the edema fluid, to be sure, but it remained in a localized area and in such tinctorial concentration that lymph draining from the region could not but have shown the color too. This happened indeed after intradermal injections of dye in the edematous skin of nephritic patients, a fact which will be brought out further on.

That the deep lymphatics were patent in the edematous skin of cardiac disease could easily be demonstrated. When a region of edematous skin stained by an intradermal injection was rubbed soon afterwards with the thumb, colored streamers promptly appeared. If the rubbing had been strong, these deep streamers looked like those appearing spontaneously in a normal leg, save for the fact that they were longer and darker.

When a stained area of skin of a patient with outspoken long standing edema was stroked slowly and firmly from the injection site toward the periphery, a retrograde passage of dye took place along the superficial lymphatics. For 10 to 12 cm. dye could be seen through the epidermis running ahead of the moving finger. In some of these instances it has also been seen in the deeper channels, forming a retrograde colored streamer. We have never been able to elicit this phenomenon in normal man, either by pressure or massage, nor for that matter in the patients themselves a few days after the edema has been done away with by therapeutic measures. It seems as if extreme dilatation of the lymphatics had rendered the valves functionless or partly so, which would account for the islands of dye described above.

In the preceding paper we have reported the formation of retrograde streamers in normal skin during the reactive hyperemia that followed

20 to 25 minutes of complete circulatory obstruction in the arms of normal subjects. We are unable to give a satisfactory explanation of the phenomenon. To test for the presence of valvular incompetence in the skin lymphatics of the patients with cardiac edema we have repeatedly produced artificial streamers by immediately massaging an injected area, and have then attempted to carry out upon the lymphatics Harvey's classical experiment which demonstrated the valvular action in veins.

The first fingers of both hands, held closely together, were pressed upon the skin at the lower end of a dye streamer artificially produced by actively rubbing a region of skin just injected. At once the first finger of the hand nearest the knee was moved upward for about 10 cm., pressing upon the skin directly over the streamer. Continuous pressure was maintained by the first fingers of both hands. In this way the colored fluid within the lymphatic was squeezed along the channel in the direction of normal lymph flow while the entrance of more colored lymph from below was prevented by the pressure of the lower hand. Unfortunately by the time these manipulations were finished enough dye had escaped from the streamers in the lymphatics to color the surrounding tissues slightly. As result the segment of skin above the lymphatic and lying between the fingers of the upper and lower hands,—which still maintained pressure,—showed some color because of the extravascular dye. While pressure was maintained over the lower end of the streamer the upper finger was removed from the skin to allow the stained lymph to return in a retrograde direction into the vessel from which it had just been squeezed. There appeared to be an increase in the color of the previously pale colored strip of skin, indicating a retrograde flow of colored lymph into the previously empty lymphatic. However owing to the obscuring presence of extravascular dye, as above mentioned, one cannot be wholly certain that a retrograde flow of the colored lymph actually occurred, though the observations strongly suggested it.

When similar tests were made on normal subjects the skin above the compressed lymphatic also became slightly tinged with blue by the time the first manipulations could be made. But when the upper finger was raised there was no observable increase in color in the previously compressed segment, that is to say no apparent retrograde flow.

In both normal subjects and cardiac patients tests were made like those just described save that pressure was maintained by the finger at the upper end of the streamer and released by that at the lower end. When, now, the injected area was rubbed, the color of the streamer returned as far as the upper finger, showing that the dye-stained lymph readily entered the lymphatics in the normal direction of lymph flow.

Postural Effects on Lymph Drainage from Edematous Skin

According to our observations therapeutic measures which relieve the edema of patients with cardiac incompetency do not notably increase the lymph flow while so doing. What are the effects of massage, mild exercise or change of posture upon the rate of flow or drainage of edema fluid by way of the lymphatics in these patients? That these procedures increase lymph flow in normal individuals (1, 6) and in animals (6-10) is well known. The preceding paper has shown too that dye streamers in normal limbs increased under these circumstances. Experiments on cardiac patients showed that slight drainage by way of the lymphatics could be obtained by active local manipulation.

We have already described the effect on dye spread of rubbing the recently injected normal skin. Dye is forced farther than usual through the minute lymphatics and soon one sees the development of colored streamers, darker and longer than normal ones. This is the case also in edematous skin. The edematous ankles of 4 patients were massaged for a few minutes after making the usual dye injection and in consequence dye was forced into the draining subcutaneous lymphatics and long streamers promptly appeared. The phenomenon will be discussed below. As our previous work has shown, such streamers of dye result from exercise of the normal leg. Five trials of the sort were made with 3 ambulatory cardiac patients having mild edema of the legs. In each, slight streamer formation took place, far less than that seen in normal limbs under the same circumstances. The patients were then allowed to rest on a high chair, feet hanging down. Almost at once the short dye streamers paled and in two instances disappeared, leaving only faint, almost invisible streaks where color had escaped from the draining lymphatics into the surrounding tissue. In normal individuals the streamers remained. It seemed as if the colored fluid which had risen in the draining lymphatics, forced up by the muscular movement, had fallen back to a lower level when this movement ceased and the limb hung vertically, the lymphatic valves then failing in their function.

As is well known, the edema of the legs occurring in cardiac disease may shift upon change in posture. Under such circumstances is there any fluid drainage by way of the lymphatics? In 2 of our cases we elevated an edematous leg while the patient remained semi-recumbent in bed and then injected dye into it. In these instances colored streamers had appeared within 6 and 10 minutes respectively after the dye injection, and within 20 minutes they had extended 10

cm. or more toward the trunk, showing that lymph flow had followed upon the postural change.

Tests were made upon 3 patients who showed transitory edema of the foot and ankle, which became evident in an hour or two on standing and disappeared within about the same period of time when the leg was once more horizontal.

Each of the patients was injected in one ankle before rising in the morning. For half an hour the behavior of the dye was studied, and as usual no streamers appeared. The patients then sat in a chair, with both feet on the floor, for an hour, after which the same ankle was again injected, and again no streamers appeared. The subjects remained thus until both ankles pitted on pressure, after which they returned to bed, with legs horizontal. After another 15 or 20 minutes, the second ankle of each was injected in the usual way.

In each instance within 20 minutes short streamers developed 5 to 7 cm. long. In length and intensity they were similar to the streamers seen in the normal, resting, horizontally placed leg at equal intervals of time and after the injection of similar amounts of dye. That is to say lymph flow, as judged by streamer formation, had been induced in the edematous legs of patients with cardiac edema. The posture would undoubtedly aid in removing the collected edema fluid.

Cutaneous Lymph Flow in Patients with Nephritic Edema

In contrast to the stagnation of lymph in the edematous skin of patients suffering from cardiac disorder, there exists a greatly increased cutaneous lymph flow in patients with nephritic edema. More than 70 tests similar to those just reported were carried out on 14 nephritic individuals varying in age from 3 to 35 years. In most the edema had been preceded or attended by a lowering of the plasma proteins. Intradermal dye injections were made in the usual manner into the skin of the ankle and occasionally on the volar surface of the forearm, 6 of the patients being injected repeatedly during the different stages of the disease, that is to say while edema was increasing, while it seemed to be stationary and during marked diuresis. In all the lymph flow was increased as judged by the rapidity and intensity of streamer formation. The lymphatic capillaries were wider than the normal but much narrower than in advanced cardiac edema. Dye entered the channels more easily, was carried further to color a wider area,

and escaped more rapidly too, but all these changes were less pronounced than in the skin of the cardiac patients.

The characteristics of the lymphatics and of the lymph flow differed much in the same individual during the various stages of his disease. The findings can best be presented by a description of typical tests upon a patient during the periods of edema increase, of fluid equilibrium and of diuresis and edema loss.

The Flow during the Stage of Marked Edema with Diuresis.—Fig. 6 (*a* to *i*) shows the result of dye injection into the ankle of an edematous, nephritic patient 2 days after diuresis had begun. For comparison with the previous figures, photographs are shown which were taken at intervals of 17, 30 and 51 seconds, 1½, 2, 5, 18, 20 and 25 minutes respectively after the beginning of the injection, which took 55 seconds.

The patient had been in the hospital for 4 weeks. He was a man 27 years old, with chronic hemorrhagic nephritis. Although confined to bed there had been a daily gain in weight with increasing ascites and edema of the legs during the first part of the hospitalization; then a period of equilibrium, and finally one of rapid loss of weight owing to profuse diuresis. On 12 occasions dye tests were made, the first 4 of them while edema was increasing and the next 3 while it seemed stationary. By good fortune the 8th injection was done just before diuresis became clinically evident, the 9th a day later when diuresis was well under way. The 10th test, now to be described, was done the following day and yielded results similar to those of the 9th test. In the 2 preceding days the patient had lost nearly 2 kilos in weight, the total plasma proteins were 3.83 per cent and the urea clearance only 14 per cent. The skin of the ankles and legs was soft and moderately edematous, pitting on pressure. The plicae, however, were far from obliterated.

On injection the lymphatic capillaries proved to be moderately widened and broader than in normal skin, yet narrower than in that of the patients with cardiac edema. The dye entered the channels readily and extended further primarily, coloring a wider area than in normal skin. These facts are shown in Figs. 6 *a* to *e*, which should be compared with Figs. 7 *a* to *d*, depicting the results of injection into the same patient when relatively edema-free about a month earlier. It will be seen that much of the dye entered the lymphatic channels directly and the interstitial bleb of colored fluid forming at the needle point was small. The staining of the superficial plexus was richer than usual, denoting free communications between the channels. Dye escaped from these channels secondarily with more rapidity than from those of normal skin, a circumstance not evident in the photographs.

Dye could be seen entering the deeper draining channels (Figs. 6 *d* and *e*) within 1/2 minute after withdrawing the needle and approximately 1½ minutes

after beginning the injection. A few minutes later streamers had formed and these grew rapidly in length and intensity, showing a far more active lymph flow than had ever been seen in normal persons or indeed in this patient prior to the onset of diuresis. The finding was characteristic of all patients injected while in the stage of diuresis. Within less than 5 minutes after beginning the injection pale streamers 6 to 7 cm. long became visible (Fig. 6 *f*). By the 6th or 7th minute these joined apparently into one band extending 4 cm. further. By the 10th minute the streamers had extended another 6 cm., forking and travelling upward yet another 3 cm. by the 13th minute, a total of 20 cm. from the center of the injection site. By the 15th minute the 2 streamers had progressed 7 cm. further up the leg and by the 18th minute had joined and had reached the knee at the level of the patella, 31 cm. from the point at which dye entered the skin (Fig. 6 *g*). Two other photographs are reproduced to show the intensity of the streamers after 20 and 25 minutes respectively. By the 28th minute the streamer could be seen at the level of Poupart's ligament, 70 cm. from the injection site.

Dye that had escaped from the lymphatics filled at the original injection spread more rapidly through the tissues than usual during the period of streamer extension. A comparison of the photographs of the normal ankle, Fig. 1 *i*, taken 20 minutes after an injection, with Figs. 6 *f* and *g*, taken at 5 and 18 minute intervals respectively, brings this point out. During this period and indeed for an hour the streamers deepened in color and broadened, owing to escape of dye from the lymphatic trunks. Thereafter they gradually paled though they were still present 3 hours later as continuous colored bands. By the 5th hour only segments of them remained visible. The dye at the injection site had spread still further interstitially and had paled greatly. The rate of disappearance of dye seemed much more rapid than in the normal subject.

It is obvious from the findings that lymph flow was far more rapid than usual. The course of events after dye injection into the other patients tested under the same conditions was the same, colored streamers extending from the ankle to Poupart's ligament with equal or greater rapidity. Indeed in one nephritic patient streamer formation was so rapid in the horizontal resting leg that a test was made forthwith to determine whether lymph flow might occur in the other leg after it had been allowed to hang vertically over the edge of the bed for 12 minutes and while still in this position. Within 15 minutes after injecting dye in the skin of the ankle streamers had extended against gravity from the ankle to the knee,—a phenomenon never seen in normal individuals with the leg hanging down. The islands of dye observed in the cardiac cases were not seen nor were other signs of lymphatic incompetence met with in any of the stages

of nephritis. The color phenomena showed a great exaggeration of the general features of the normal.

Lymph Flow during the Formation of Edema and in the Periods of Relative Equilibrium of the Fluids.—When edema was in process of forming or while it remained stationary the differences from the normal were less apparent. Tests made upon the patient who furnished the data just given showed these points typically.

One month prior to the experiment now to be described edema of the ankles was just detectable by pressure of the thumb. The skin was soft and pale, and the plicae not obliterated. Nevertheless edema was increasing, for the patient gained 1/4 kilo each day. The plasma contained but 1.00 per cent albumin and 2.08 per cent globulin, yielding an A/G ratio of only 0.48.

Dye injection showed the lymphatic capillaries to be less dilated than during the period already dealt with; the superficial plexus was far less extensively injected; and the interstitial bleb at the end of the needle was larger and more like that formed in normal skin. Nonetheless the dye passed with unusual rapidity into the draining trunks. In Figs. 7 *b*, *c* and *d*, taken 30, 40 and 51 seconds after the beginning of an injection, which required 1 minute to complete, faint cloudy extensions of dye can be seen in the deeper draining channels, becoming much more obvious in *e* and *f*, which were taken 1½ and 3 minutes respectively after beginning the injection. Dye escape from the small lymphatic capillaries was rapid, their individual outlines soon becoming merged in a blur of color. In Fig. 7 *g*, the arrow at the left in the photograph marks the upper extremity of the dye streamer 20 minutes after beginning the injection. It was easily visible to the eye and longer than the average colored streamer found in normal ankles at rest, but streamers of like dimensions have been seen from time to time in tests upon normal individuals. The second arrow indicates the course of the streamer. 2 other injections in the same patient 2 and 3 days later yielded similar results. The difference between this result and that obtained during diuresis, as already described, was pronounced.

For more than a week after this test the patient's edema increased and then became approximately constant for another week. At this time the legs were markedly edematous, the skin soft and the plicae partially obliterated. Deep pitting showed itself on pressure. Dye injection into the skin of the anterior surface of the ankle at the level usually selected for such injections showed the lymphatic capillaries more dilated than in the period of edema increase just described. The findings were similar to those shown in Figs. 6*a* and *b*. Dye escaped from the superficial lymphatics more rapidly than from those of normal skin but one cannot say that escape was more or less rapid than in other tests upon this patient. As in the test first described (Fig. 6), the lymphatic capillaries took up the dye readily and it colored a wider area than that shown in Fig. 7. As result the dye bleb at the needle point was not as large as that seen when normal skin is injected. During the injection itself, which lasted but ¾ minute, dye passed

into several draining trunks, and within 2½ minutes streamer formation began, becoming pronounced within 3 minutes. The streamers darkened faster than under normal conditions and by the 8th minute extended 10 cm. up the leg. By the 15th minute they had outstripped the streamer shown in Fig. 7, being 18 to 20 cm. long. After 20 minutes they were easily detectable about 25 cm. above the site of injection, that is to say about 12 cm. below the patella. The streamer formation was more rapid and apparent than in the previous week when edema was increasing, but not as great as in the period of diuresis.

Other studies on nephritic patients have given similar results. The variations from the normal were evidently determined by the stage or severity of the disease process.

A few atypical findings deserve notice.

A dye injection was made as usual into the ankle of an obese woman of 25 years with generalized edema of face, trunk and extremities and tense ascites. The patient had pronounced glycosuria and mild nephrosis, with the plasma proteins reduced but moderately, to 4.5 per cent. Her feet and legs were fat and enormously swollen, the skin, glistening and soft, pitted easily on pressure. The plicae were obliterated. There was no evidence of cardiac decompensation. On test the lymphatics were found widely dilated, and the injected dye entered them with great ease. The resulting picture was in one respect similar to that seen in the edema of long standing cardiac disorder, islands of dye appearing, but streamer formation occurred. A pale extension of dye running up the leg for 6 to 7 cm., and requiring nearly half an hour to form, yielded evidence of a little lymph flow, far less than the normal. During the test the patient remained semirecumbent in bed, the abdomen pressing upon the skin of the upper legs. The test was repeated 2 days later with the patient lying flat on the back to avoid pressure upon the legs. Streamer formation was much more active, 2 streamers 20 cm. long developing in 20 minutes. The new posture presumably relieved lymphatic obstruction.

Sometimes the test has yielded unexpected results that were highly informative. One case should be cited but without undue emphasis.

For a week the nephritic patient, C, whose tests have already been described, had been in a stage of unchanging edema. One more test in this period was desired for purposes of comparison. Shortly after the injection streamers developed which lengthened and darkened with unexpected rapidity, resembling those appearing in other nephritic patients during periods of diuresis. The urine collections of the next 12 hours showed that diuresis actually had set in. 24 and 48 hours later, while diuresis was at its maximum, the 9th and 10th tests were made (Fig. 6), which produced long dye streamers that have already been described.

One is tempted to infer from this case that the method used is sufficiently delicate to elicit evidence of fluid movement through the lymphatics at the onset of diuresis before the latter becomes certain from urinary measurement. We feel, however, that individual differences are too large for such findings to be reliable. Recently Aldrich and McClure (11) have studied the rate of disappearance of saline blebs injected subcutaneously in patients with edema and they have reported that, in these cases, just prior to the onset of diuresis, the bleb fluid is removed more rapidly than during periods of stationary edema; and still more rapidly when diuresis is established. No doubt the two phenomena have something in common.

DISCUSSION

Before discussing the results it may be well to appraise the method employed. The evidence that the length and intensity of the colored streamers can be interpreted in terms of lymph flow has already been given (1). However, intradermal injections of vital dyes are subject to many influences, and these must be reckoned with. A faulty insertion of the needle, so that it goes too deep in the skin, or on the other hand an especially fortunate injection, whereby all the dye is introduced directly into the lymphatics, may prove misleading. To avoid these sources of error we have injected the patients 2 or 4 times upon each occasion, and in studying changes in lymph flow at the various stages of a disease have injected the patients upon several occasions during each stage. For example the nephritic patient yielding the results already described received 2 to 4 injections on each of 12 occasions,—3 or 4 of these in each stage of his disease. The tests yielded notably concordant results. In all instances the injections were made on the anterior surface of the ankle at approximately the level of the malleoli of the tibia, as close to the midline as possible. Slight variations in the level of the injection site to avoid recently injected areas or skin blemishes have yielded no significant differences in the tests upon either normal subjects or patients.

The previous activity and posture of the limb have much to do with the formation of streamers, as shown in the preceding paper. One cannot state categorically that lymph flow in the edematous skin of the leg of nephritic patients is greater than the normal at all times.

Such a statement might prove erroneous if lymph flow of the bed-ridden patient was compared with that of a normal individual during periods of muscular activity or with that in the skin of a normal limb recently elevated from a dependent position and in which in consequence lymph drainage was taking place with unusual rapidity. Yet when such a comparison is made lymph flow in the edematous skin of the resting nephritic patient usually proves the greater. We can state with assurance, however, that there is no sign of sub-normal lymph flow during the building up of edema in the nephritic patient, while there is clear evidence of an increase in flow during the periods of edema equilibrium or subsidence. In the edematous skin of the horizontally resting legs of the cardiac patients on the other hand lymph flow is negligible.

The lymphatics in edematous skin are open. Even during the tense edema of cardiac disease they are found to be wide and full of fluid. We have already shown that such is their condition in the ear of the mouse (5) rendered edematous by paintings of xylol; and more recently Pullinger and Florey (12), using the same organ, have shown that local edema following injection of saline or 1 to 1,000 histamine solution into the tissues leads to dilatation of the lymph channels. Clark and Clark (13) have shown that lymphatics in edematous tadpoles are dilated and filled with fluid, and Drinker and Field (6) report that swelling of the tissues after complete venous blockage does not shut off large collecting lymphatics. Why, then, do the lymphatics function so well in edematous skin of nephritic edema and scarcely at all in that of cardiac patients?

In the nephritics tested the blood pressure was normal or only slightly raised; hypertension cases were avoided. There were no obvious signs of cardiac weakness, and the concentration of plasma proteins was much decreased. It follows that there was an increased escape of fluid from the blood capillaries of these patients, together with a more active circulation than obtained in the cardiac patients. This difference readily explains the increased fluid turnover by way of the lymphatics. In advanced cases of nephritis, in which the colloid osmotic pressure of the blood might on occasion be lower than the capillary hydrostatic pressure, we would expect to find even more of the fluid, normally returned by the blood, taken away by the lymphatics.

The stagnation of lymph in the edematous skin of the cardiac patients is not easily explained. In cardiac incompetence the venous pressure is generally greater than normal. Can the higher pressure in the veins at the point where thoracic duct lymph enters the blood be transmitted to the peripheral lymphatics and account for the stasis of lymph? 4 tests were made on this point. Patients with cardiac failure and edema of the ankles and legs but none of the arms were so placed in bed that the wrists and ankles lay at the same level. In this position any back pressure existing must have been the same in the lymphatics draining the upper and lower limbs. Yet while dye injections in the arms resulted in the development of colored streamers with the rapidity and intensity seen in normal subjects (as described in the preceding paper), that introduced into the skin of the ankles gave rise to no streamers. Lymph flow in the arms appeared to be normal whereas in the legs it was absent, a finding which would appear to rule out decisively the influence of back pressure to account for the stasis of lymph.

A partial failure in the circulation of blood cannot explain the complete cessation of lymph flow in the cardiac cases, though it might account for a decrease in lymph formation. Both the cardiac and nephritic patients showed dilatation of the lymphatic capillaries, the former far more than the latter. In both more dye entered the lymphatics directly during the injection than was the case in normal skin. Yet in the cardiac patients it was not drained away. Stagnation of lymph might be accounted for by a change in the permeability of the lymphatics so great that fluid within these vessels might pass without resistance into the large reservoir of edema fluid; but a change of this magnitude is not indicated by our findings. To be sure, the lymphatic capillaries of both cardiac and nephritic patients were more permeable than normal, but the observed differences in permeability were quite similar in both, judging from the rapidity of dye escape, and surely they were not great enough to account for the absence of streamer formation in the edematous legs of the cardiac patients through the escape of dye from the channels before the current of lymph had swept it along. Nor can it be assumed that streamers failed to develop in the legs of these patients because the dye was diluted to invisibility by the great amount of fluid present. For in the edematous skin of nephritic patients dye escaped with approxi-

mately equal speed into the tissue fluid, yet streamers of color developed in the deeper lymphatics which were longer and more deeply colored than in normal limbs, the increased amount of fluid present having no evident effect to prevent their formation.

The presence of retrograde streamers in the edematous skin of the cardiac patients and their absence from that of the nephritics constitutes evidence for the existence of a valvular incompetence of the lymphatics in the patients of the sort first mentioned. The presence of such a valvular incompetence would explain the lack of lymph flow, but, as already described, complete proof of its existence is lacking.

SUMMARY

Local intradermal injections of dye have disclosed the fact that the skin lymphatics in regions of cardiac edema are patent, full of fluid and much widened. Intercommunication between them is ready and dye escapes from them more rapidly than from the vessels of normal skin. A retrograde distribution of dye by way of the lymphatics often occurs and it may pass unseen along the deeper channels to emerge in the skin at unexpected, distant situations. A valvular incompetence of the lymphatics consequent on dilatation would appear to be the cause of these phenomena. In regions of cardiac edema lymph stagnates, despite the fact that the channels are open.

In nephritic edema the lymphatic capillaries are wider than normal but not as wide as in cardiac edema. No sign of incompetency of the valves is to be observed. Instead lymph flow is considerably greater than normal, even when edema fluid is accumulating. It was noted to be greater in the periods of fluid equilibrium also and extraordinarily rapid in periods of diuresis.

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EXPLANATION OF PLATES

PLATE 14

FIGS. 1 a to h. Photographs of the intradermal spread of dye in the skin of a normal ankle, natural size. They were taken at intervals of 10, 16, 25, 40 seconds, 1 minute and 1 second, 1 minute 50 seconds, 2 minutes 45 seconds, and 3½ minutes respectively after beginning the injection, which lasted 62 seconds. The interstitial bleb of dye which formed at the point of the needle is of about the size usually seen. Dye escape from the lymphatic capillaries colored with the dye appears as a fuzzing at their borders. Fig. e is the last picture to show the injecting needle.

FIG. 1 i. Taken 20 minutes after making the injection. 2 pale streamers of dye approximately 10 cm. long had developed in this period. Reduced to 1/7 natural size.

FIG. 2. Spread of dye in the edematous skin of the ankle of a patient suffering from cardiac insufficiency. The natural size photographs were taken at 10, 15, 25 seconds respectively after beginning the injection, which lasted only 37 seconds. Fig. 2 d, composed of parts of four overlapping photographs taken at 10 second intervals from ¾ of a minute to 1½ minutes after beginning the injection, shows the size of the injected area. The lymphatic capillaries are seen to be widely dilated and dye escape from them has been rapid. Compare with Fig. 1. Fig. 2 f, taken 20 minutes later, and reduced to 1/7 natural size, shows the large area covered by the injection and the absence of deep streamers of dye.

PLATE 15

FIG. 3. Results of a dye injection into the skin of the same patient, who was now rapidly losing edema. The photographs were taken after intervals of 18, 25, 35 and 55 seconds, 2 minutes 20 seconds and 20 minutes from the beginning of the

injection, which lasted 50 seconds. Note that almost all the dye is within lymphatic capillaries: there is almost no interstitial bleb. Again no deep, colored streamers can be seen. Magnifications similar to those of Fig. 2. The central pale areas in these and other photographs are high lights caused by dye solution that had escaped on the surface of the skin.

FIG. 4. Results of an intradermal dye injection into the edematous skin of another patient with cardiac edema. Photographs *a* to *e* inclusive (natural size) were taken at intervals of 20, 30, 55 seconds, $1\frac{1}{2}$ and $3\frac{1}{2}$ minutes respectively after beginning the injection, which required 48 seconds. Photograph *f*, reduced to $1/7$ natural size, was taken 20 minutes after beginning the injection. Islands of dye are shown, as described in the text. Again no streamers were visible. In photographs *c*, *d* and *e* the shiny, white marks in the center are due to high lights caused by dye solution on the surface of the skin.

FIG. 5. The same phenomenon as Fig. 4, but as it appeared half an hour after beginning the injection, which lasted less than a minute. The figure, described fully in the text, is about $1/2$ natural size.

PLATE 16

FIG. 6. Results of an intradermal dye injection in an edematous, nephritic patient during a period of flood diuresis. The injection required 55 seconds. The photographs were taken at intervals of 17, 30, 51 seconds, $1\frac{1}{2}$, 2, 5, 18, 20 and 25 minutes respectively after beginning the injection. The first five are natural size. Fig. 6 *f* is reduced to $1/7$, Fig. *g* to $1/11$, Figs. *h* and *i* to $1/5$. The lymphatic capillaries are wider than in normal skin but less so than in the skin of cardiac edema; the injected area is large, dye escape rapid (Figs. 6 *a* to *d*) and the interstitial bleb of dye small. Beginning streamers appeared early, after $1\frac{1}{2}$ to 2 minutes (Figs. 6 *d* and *e*) and streamers 6 to 7 cm. long developed in less than 5 minutes (Fig. 6 *f*). Note the strong bands of color showing at the level of the knee (Fig. 6 *g*), in the photograph of the same leg taken after 18 minutes but reduced to $1/11$ of the natural size. The leg was rotated slightly to show the intensification of the lower streamer. The two larger pictures are reproduced to show the remarkable streamers.

FIG. 7. Results of an injection of dye like that of Fig. 6 and made in the same patient during a period in which edema was increasing. The photographs were taken 18, 30, 40, 51 seconds, $1\frac{1}{2}$, 3 and 20 minutes after beginning the injection, which required 1 minute to complete. The first five photographs are natural size; the last is reduced to $1/7$. The lymphatic capillaries are much more normal in appearance than in Fig. 6, but streamers soon began to appear. In 20 minutes the streamer flowing from the injected area reached the point indicated by the upper arrow. The tip of the streamer was too faint to be discernible in the photograph.

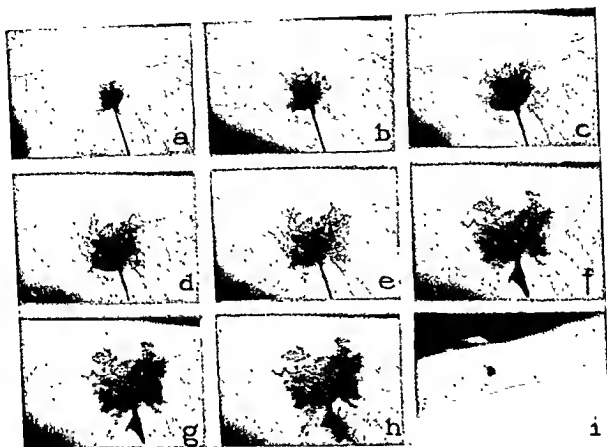


FIG. 1

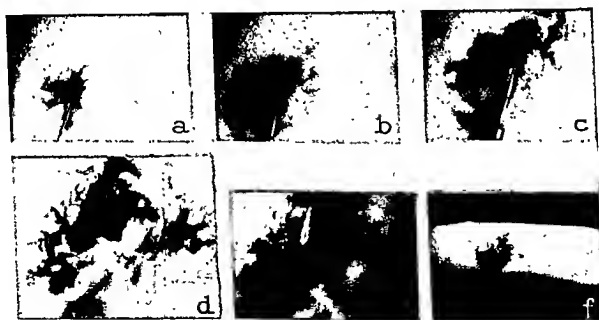


FIG. 2

(McMaster, Lymphatics and lymph flow in edematous skin)

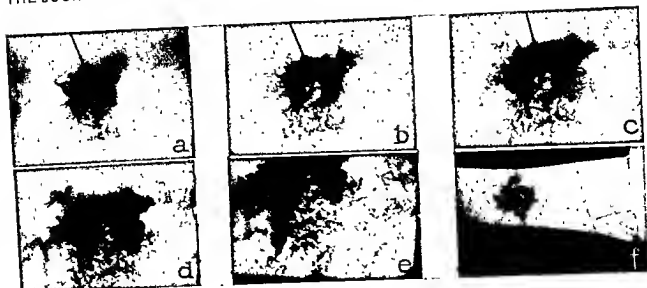


FIG. 3



FIG. 4

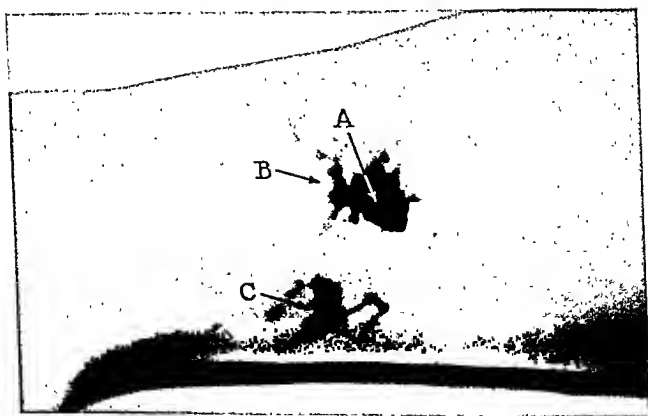
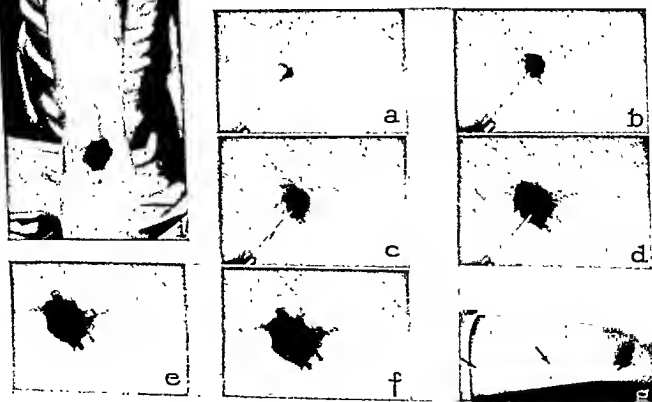


FIG. 5





FIG. 6



THE MOLECULAR WEIGHT OF ANTIBODIES*

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It is now generally accepted that antibodies are actually modified serum proteins. The work of Felton on the concentration and nature of pneumococcus antibodies (1) and a series of quantitative analytical studies on the precipitin reaction and its mechanism (2) have contributed to the adoption of this conclusion, and have recently led to the isolation of serum protein fractions of which up to 98 per cent of the protein present could be accounted for as antibody by actual chemical analysis (3). The availability of material of such high antibody content suggested the present study.

The determination of the molecular weight of antibodies is of interest in a number of connections. Knowledge of this constant would be expected to throw light on the relation of antibodies to normal serum proteins and on the mechanism of antibody formation. Such a study should also permit chemical formulas to be written for some of the limiting compounds formed in antigen-antibody combination, a subject which will be taken up in another communication. Finally, should differences be found in antibodies produced by different species of animals, this would not only be of physiological interest but might also be of importance for serum therapy. Preliminary studies on antibody particle size have already been reported (2d,¹

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** Recipient of a partial fellowship from the John Simon Guggenheim Memorial Foundation.

¹ Heidelberg and Kendall (2 d), page 570.

and 4-7) and in the present communication details of our experiments (7) are given.

In this study sedimentation constants were determined in the Svedberg ultracentrifuge (8), in which centrifugal force at high rotational speeds overcomes diffusion and permits the evaluation of particle size from the rate of sedimentation. The material used included whole normal sera and antisera, normal globulin, immune globulin containing up to 50 per cent of anti-egg albumin, Type I pneumococcus antibody concentrate prepared from horse antisera by the Felton method (1), and pneumococcus anticarbohydrate containing up to 98 per cent of antibody, prepared as in Reference 3 from horse and rabbit antisera.

EXPERIMENTAL

Methods

In all but one instance (Experiment 7) the Lamm scale method (9), modified as described by McFarlane (10) and one of us (11), was used for recording the course of sedimentation in the ultracentrifuge. The projection system used was that described previously (11).² With this method an equidistant scale is photographed at intervals through the rotating cell. These photographs are compared microscopically with those from a reference scale (11) obtained from a run under similar conditions but with the cell filled with the buffer or salt solution used in the actual run. The displacements of the scale lines, Z , from their positions on the reference scale are plotted as ordinates against the corresponding positions in the cell as abscissae (distance from the axis of rotation) yielding a curve like that in Fig. 1, which shows the sedimentation diagram for a mixture of serum albumin and lactoglobulin. If the resolving power of the centrifuge is sufficient and if the sedimentation constants are not too similar, each particle size will be represented as a peak on the sedimentation diagram as in Fig. 1, in which the heavy line gives the experimental values, and the broken lines indicate in part the two individual curves which give rise to the heavy curve. Since

$$Z = k \cdot \frac{dc}{dx}$$

where k depends on the refractive increment of the sedimenting substance and on known apparatus constants, it is evident that integration of the entire individual curve corresponding to a single peak will give the concentration, c , of the substance having the sedimentation constant of that peak. From the sedimentation

² Pederson (11), page 48.

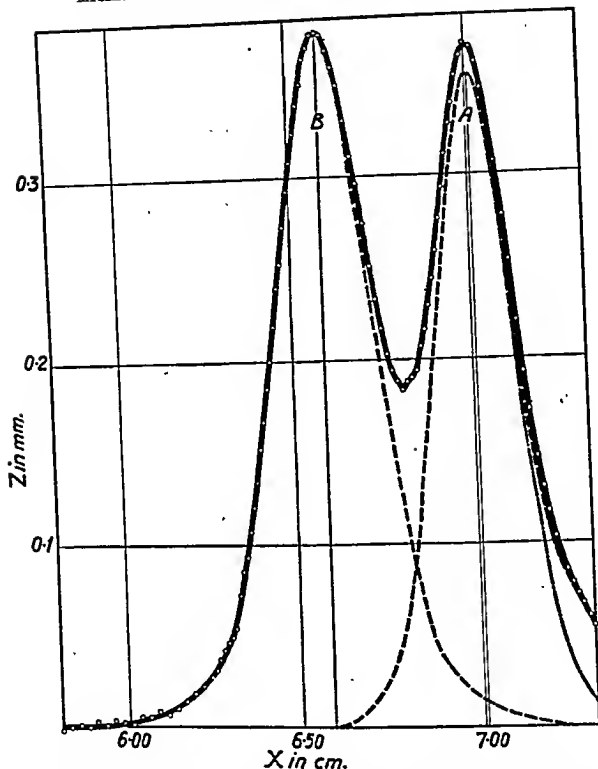


FIG. 1. Analysis of mixture of serum albumin (A) and lactoglobulin (B) in the ultracentrifuge. The experimental curve (solid line) was analyzed to give the individual curves for each component (broken lines). The sedimentation diagrams have been analyzed similarly in the present study.

diagram obtained by the Lamm method one thus obtains both the sedimentation constants (from the change in position of the peaks with time) and the concentrations of the molecules in the solution under investigation. The following

values were used for the refractive index increment, α , at wave length $\lambda = 436 \text{ m}\mu$: for egg albumin, $\alpha = 0.001885$; for serum albumin, $\alpha = 0.001947$; for serum globulin, $\alpha = 0.001967$ (12).

All runs were made at 51,000 and 59,000 R.P.M., corresponding to 190,000 and 250,000 times gravity, respectively. The higher speed was used for most of the rabbit material.

All sedimentation constants were corrected for density and viscosity in the usual way, and in addition for the viscosity due to the protein itself (von Mutzenbecher (13)), since otherwise the sedimentation constants for the faster sedimenting molecules are not comparable.

Except for Experiment 7, all sera and antibody solutions were dialyzed in cellophane against 0.2 M sodium chloride solution, under pressure when concentration of the solution was necessary. Nitrogen analyses were run on all solutions by the micro Kjeldahl method. Values for milligrams of total N per milliliter were transformed to grams of protein per 100 ml. of solution by multiplication by 0.632 for the serum proteins and 0.645 for egg albumin. The precipitin content of all antisera and antibody solutions was determined by the absolute methods given in References 3 and 2*b*.

Rabbit serum globulin was prepared for comparison with whole rabbit sera by dilution of 5 ml. of serum with an equal volume of water and addition of 9 ml. of sodium sulfate solution saturated at 37°C. After centrifugation the precipitate was taken up in 10 ml. of warm sodium sulfate solution at the final concentration and again centrifuged. The globulin was finally taken up in 5 ml. of water and run through a small Chamberland L2 filter.

The Felton solutions (1) were prepared by pouring horse serum into 20 volumes of chilled 0.01 M phosphate buffer at pH 5.5 (calculated), collecting the precipitate, and redissolving it in 0.9 per cent sodium chloride solution.

The dissociated antibody solutions were prepared by dissociation of the washed specific precipitates with strong salt solution (3), and in one instance with barium hydroxide and barium chloride (3).

The analyses and the experimental results from the centrifuge runs are summarized in the tables and Figs. 2 and 3. The latter show the relative distribution of the amount of substance used with respect to particles of the sedimentation constants determined from the sedimentation diagrams. The area of the rectangles is proportional to the relative concentration of the molecules having the given sedimentation constant. These rectangles also indicate whether the corresponding peak in the sedimentation diagram is almost homogeneous or not, since their breadth is put equal to a sedimentation constant unit ($1 \cdot 10^{-13}$) for the homogeneous peaks, whereas the non-homogeneous peak is indicated by a broader rectangle. The letters *c* and *s* give, respectively, the concentration in gm. per 100 ml. of solution and the sedimentation constant times 10^{13} for the given component. In Figs. 2 and 3 the rectangles corresponding to egg albumin, serum

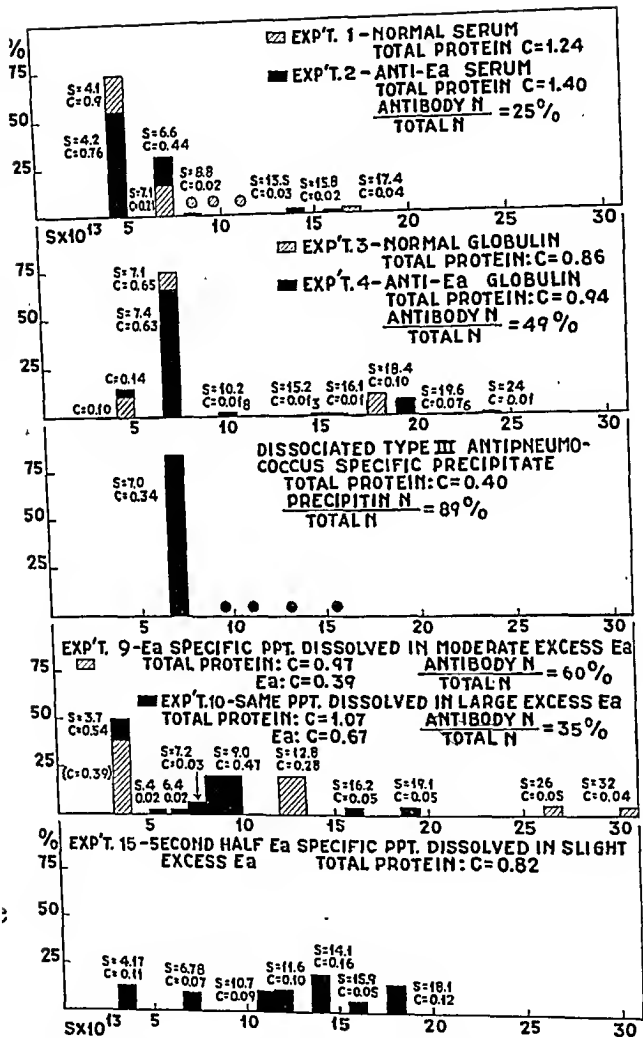


FIG. 2. Quantitative diagrammatic representation of molecular species found in ultracentrifuge runs on rabbit sera and antibody solutions. Explanation at bottom of page 396.

albumin, and serum globulin (Ea, Sa, and Sg³) are always placed at the normal s value for these substances, whereas for all the other substances studied the rectangles are placed at the experimentally determined s value. The error in the determination of the absolute s value for the faster sedimenting molecules is generally ± 5 per cent and may occasionally increase to ± 10 per cent in the most complicated diagrams. For the homogeneous solutions the errors in s are smaller. Circles in the diagrams indicate inhomogeneous material.

Observations on Rabbit Sera and Antibody Solutions

Experiment 1. Sterile Normal Rabbit Serum 4.36₀ Containing No Added Preservative.—This serum contained 10.4 mg. total N per ml. After an arbitrary deduction of 0.3 mg. for non-protein N the serum contained 7.2 mg. albumin and 2.9 mg. globulin per ml. by the Howe micro method (14). Thus 69 per cent of the total N was due to albumin and 28 per cent to globulin. The concentrations calculated from the sedimentation diagram were 73 per cent⁴ of albumin, in good agreement with the analytical value, while the globulin component was only 17 per cent of the total, or considerably less than the analytical value. However, the sedimentation diagram also showed the presence of several small peaks which partially explain the low value for the normal (principal) globulin peak. It has been found by one of us⁵ and by von Mutzenbecher (13) that normal globulin always contains small amounts of molecules with s about $18 \cdot 10^{-13}$. This serum, too, showed the presence of molecules with $s = 17.4 \cdot 10^{-13}$ (3 per cent). The ratio of the concentration of the principal globulin component to that of the 17 component was 5.7. Further details of the run are to be found in Fig. 2*a*.

Experiment 2. Sterile Rabbit Anti-Egg Albumin Serum 4.36₂ Containing No Added Preservative.—This serum was obtained from the same rabbit as the normal serum, after two courses of intravenous injections, using 5 mg. of alum-precipitated Ea per injection (*cf.* 2*c*). Of the 11.7 mg. of protein N per ml. of the serum, 6.1 mg. were globulin N and 5.6 mg. albumin N (Howe method). The serum contained 3.1 mg. of anti-Ea N per ml., or 26 per cent of the protein N. The sedimentation diagram also showed a strong increase over the normal serum in the globulin peak and a corresponding decrease in the other peaks. The analysis gave 48 per cent for albumin, while the sedimentation diagram gave 54 per cent. For the globulin the values were 52 and 31 per cent, respectively, in poor agreement. The ratio between the amounts of the 7 component and the 17 component was 22; that is, the increase in the principal globulin component was greater than in the smaller, heavier one (see Fig. 2*a*).

³ These designations are also used for these proteins throughout the experimental part.

⁴ All subsequent values are given as per cent of the total protein calculated from the amount of total N present.

⁵ Pedersen, K. O., unpublished results.

Experiment 3. Normal Rabbit Globulin 4.36₀.—The solution was obtained from the serum used in Experiment 1 as previously described. The sedimentation diagram showed 76 per cent of the principal globulin, 11 per cent of albumin, and, in addition, 12 per cent of a component with $s_{20} = 18 \cdot 10^{-13}$ and other heavy components in low concentration. The ratio of the normal globulin to the 18 component was 6.5 (Fig. 2b).

Experiment 4. Immune Rabbit Globulin 4.36₂.—This was obtained from the anti-Ea serum used in Experiment 2. Although 50 per cent of the protein present was specifically precipitable by Ea the sedimentation curves were scarcely different from those of the corresponding normal globulin. The ratio between the normal and the faster sedimenting globulin was 8.3, a small increase (Fig. 2b).

Experiments 5 and 6. Immune Globulin.—These experiments on immune globulin obtained from two other anti-Ea sera, 4.37₂ and 4.31₂, gave essentially the same result as Experiment 4. The ratio between the two globulin components in both experiments was 12.

Experiment 7. Rabbit Type III Pneumococcus Anticarbhydrate 3.50₁.—The water-clear solution was prepared by salt dissociation according to Reference 3 from the serum of rabbit 3.50, which had been injected with formalinized Type III pneumococcus. The method yields only a portion of the total antibody. The solution was preserved with 0.01 per cent of merthiolate. Although 90 per cent of its protein was precipitable by Type III pneumococcus specific polysaccharide it showed $s = 7.0 \cdot 10^{-13}$, the value characteristic as well for the principal component of normal rabbit globulin. The light absorption method was used for this run.

Experiment 8. Rabbit Type III Pneumococcus Anticarbhydrate 3.51₁.—The water-clear solution (3) was prepared from the serum (preserved in the cold with 0.01 per cent of merthiolate) of another rabbit, and 89 per cent of its protein was specifically precipitable by the homologous specific polysaccharide. It was homogeneous (84 per cent of the analytical value)⁶ in the ultracentrifuge, showing only the component $s_{20} = 7.0 \cdot 10^{-13}$. The few minor peaks present were so small that no sedimentation constants could be calculated (Fig. 2c).

The following experiments were carried out in attempts to gain information regarding the size of the molecules in the inhibition zone of the precipitin reaction, in which specific precipitation is inhibited by an excess of antigen.

Experiments 9 and 10. Egg Albumin-Anti-Egg Albumin Specific Precipitate Dissolved in Excess Egg Albumin Solution.—8.5 ml. of serum 4.36₂, preserved with 0.01 per cent of merthiolate, were divided into two equal portions, diluted with saline, and precipitated with a total of 1.73 mg. of Ea N, leaving a slight excess

⁶ Five determinations from the sedimentation diagrams agreed within 4.5 per cent.

of antibody to insure the presence of all added Ea in the precipitate (2e). After three washings with 10, 5, and 5 ml. of chilled saline the specific precipitates were recombined, suspended in 3.0 ml. of 0.2 M NaCl solution, and treated with 0.5 ml. portions of Ea solution (2.59 mg. Ea N per ml.) at 5 to 10 minute intervals, until only traces of insoluble material remained, clearing taking place when 4.0 ml. of Ea solution had been added. It was found best to warm the mixture at 35–38°C. in the presence of a drop of toluene, as solution of the precipitate required a number of hours at 0°. The solution was transferred quantitatively to a cellophane tube, dialyzed against several changes of 0.2 M NaCl solution, and made up to 10.0 ml. with 0.2 M NaCl solution. For Experiment 9, part of the solution was diluted 1:1 with 0.2 M NaCl solution, for Experiment 10, 2.0 ml. were mixed with 0.50 ml. of Ea solution containing 4.32 mg. Ea N, again dialyzed against the salt solution, and diluted 1:1 as before. Since the amount of Ea N in all samples was known, total N – Ea N = antibody N present.

The sedimentation diagram from Experiment 9 (Fig. 2*d*) showed as the main peak (besides that due to Ea) a rather inhomogeneous one with a mean s of about $13 \cdot 10^{-13}$ (29 per cent of the analytical protein value). There were also several other peaks with higher values, the two largest showing $s = 26 \cdot 10^{-13}$ (5 per cent) and $32 \cdot 10^{-13}$ (4 per cent). Experiment 10, in which a large excess of Ea was added, gave a quite different sedimentation diagram (Fig. 2*d*). The main peak was even less homogeneous and showed a lower mean s , about $9 \cdot 10^{-13}$ (44 per cent). The peak was very broad and of a peculiar shape with a sharp limit at the slower sedimenting side. This sharp boundary sedimented at the rate of normal globulin ($s_{20} = 7.2 \cdot 10^{-13}$). Most of the faster sedimenting peaks had disappeared from the diagram. This change may be explained by recent studies (15) which indicate that a lighter component in relatively high concentration causes a shift toward lower sedimentation constants for the heavier components present, a change considered as a dissociation of the heavier components.

Experiments 11, 12, 13, 14, and 15.—These experiments represent similar runs, except that in Experiments 11, 13, and 14 approximately one-half of the antibody in a portion of serum 4.36₂ was precipitated with Ea and redissolved in different amounts of Ea, while the remainder of the antibody in the serum was precipitated for Experiments 12 and 15 and dissolved in excess antigen. The experiments confirmed the previous ones in so far as a low s (but greater than that of globulin alone) was found in the experiments with high Ea concentrations, and high s values were found with low Ea concentrations. The influence of the Ea is best seen from Table III.

The behavior of the antibody was noteworthy in Experiment 15, in which the Ea content was kept at the minimum. The sedimentation diagram (Fig. 2*e*) showed a number of sharp, well defined peaks which appear due to molecules of very definite size, possibly formed by the combination of antigen and antibody in simple proportions. If further work should show this to occur with regularity it would be necessary to assume that a larger excess of Ea dissociates the original soluble complexes of large size, converting them into compounds of lower molecular

weight. There would thus be an accord between Pedersen's explanation (15), the ideas expressed in References 2 and 16, and Marrack's views (17).

Experiment 13 was a repetition of Experiment 11 on another portion of the solution 3 days later to establish whether or not the s value, higher than that of antibody globulin itself, was due to a relatively slow disaggregation of the precipitate. The value obtained, however, was not lower.

Observations on Horse Sera and Antibody Solutions

Experiment 16. Felton Solution from Normal Horse Serum.—The slight precipitate formed on pouring normal horse serum into 20 volumes of chilled 0.01 M phosphate buffer at pH 5.5 (1) was redissolved in 0.9 per cent NaCl solution and let stand in the cold for several days with a little toluene. A portion of the clear supernatant was used for the centrifuge run after dialysis against 0.2 M NaCl solution. As opposed to the Felton solutions from antipneumococcus sera (see below), very little of the component $s = 18 \cdot 10^{-13}$ was present, the main component (60 per cent, not homogeneous) showing $s_{20} = 8.1 \cdot 10^{-13}$ (Fig. 3 a).

Experiment 17. Felton Solution from Type I Antipneumococcus Horse Serum.—This was prepared as in Experiment 16 from a sample of sterile, unpreserved serum.⁷ The serum contained 0.64 mg. of Type I anticarbohydrate N per ml., the low antibody content being perhaps responsible for the relatively small proportion (29 per cent) of antibody in the Felton solution and also (>51 per cent) in the dissociated antibody used in the next experiment. Before use a portion of the anti-C was removed from the serum by precipitation with pneumococcus C substance (18). Besides several small peaks the sedimentation diagram showed two main components, one with $s_{20} = 6.8 \cdot 10^{-13}$ (40 per cent) and another with $s_{20} = 18.3 \cdot 10^{-13}$ (42 per cent) (Fig. 3 a).

Experiment 18. Dissociated Type I Pneumococcus Anticarbohydrate.—An antibody solution was prepared by salt dissociation of the washed precipitate (3) from the above serum and Type I pneumococcus specific polysaccharide (19) (referred to below as S I). Only a portion of the antibody is recovered by this method. Owing to an accident the analysis for precipitin could be made only on a portion of the solution which had been exposed to deterioration through extensive manipulation, the value obtained being 51 per cent of the total N. The dissolved protein was almost homogeneous in the ultracentrifuge, showing $s_{20} = 18.5 \cdot 10^{-13}$ (64 per cent of the analytical value).

Experiment 19. Dissociated Type I Pneumococcus Anticarbohydrate.—The solution used in this experiment was obtained similarly from a later bleeding of the same horse, the serum containing only 0.43 mg. of anticarbohydrate N per ml. The precipitin N content of the solution was 61 per cent of the total and the sedimentation diagram showed practically only a single component of $s_{20} = 18.2 \cdot 10^{-13}$ (93 per cent of the total protein (analytical)) (Fig. 3 b).

⁷ This serum was kindly supplied by Dr. S. Gard of the Statens bakteriologiska Laboratorium, Stockholm, to whom we wish to express our thanks.

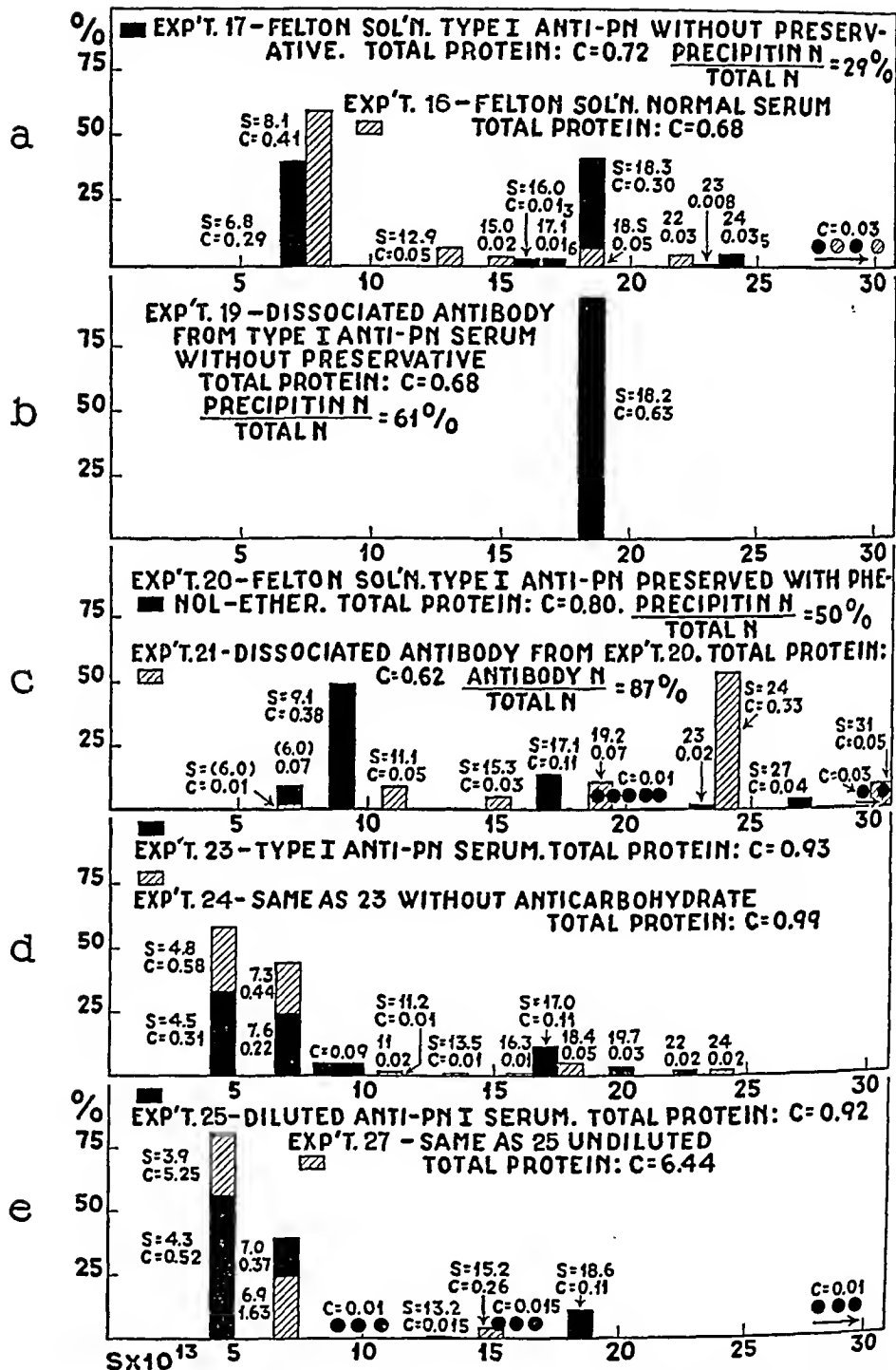


FIG. 3. Quantitative diagrammatic representation of molecular species found in ultracentrifuge runs on horse sera and antibody solutions. Explanation at bottom of page 396.

Experiment 20. Felton Solution from Type I Antipneumococcus Serum Preserved with Phenol and Ether.—The serum, No. 610,⁸ contained 1.50 mg. of Type I anticarbohydrate per ml. Before use it was absorbed with C substance and pneumococcus protein. The Felton solution was prepared as in the previous instances and 50 per cent of its protein content was precipitable by S I. In the sedimentation diagram the main component (48 per cent) was very inhomogeneous and had a low s , namely $9.1 \cdot 10^{-13}$. Several other faster sedimenting components were present, the largest having $s_{20} = 17.1 \cdot 10^{-13}$ (14 per cent), but even these were not as homogeneous as in the preceding Felton solution, possibly owing to the exposure of the serum proteins to phenol and ether during storage. This would be in accordance with McFarlane's findings (20) with alcohol-ether-treated serum.

As soon as the 17 component had reached the bottom of the cell the centrifuge was stopped and the upper portion of the cell contents was pipetted off. This contained a small amount of heat-coagulable protein but gave only a faint opalescence with a 1:20,000 solution of S I. The bottom layer in the cell, however, gave heavy specific precipitation. The antibody function thus occurs with the heavier components.

Experiment 21. Dissociated Type I Pneumococcus Anticarbohydrate from Felton Solution of Experiment 20.—Although 87 per cent of the protein in this preparation (79 A₁, Reference 3) was specifically precipitable, the material was polydisperse, again possibly owing to the long exposure of the serum proteins to phenol and ether. The s of the main component (53 per cent) had in this case increased to $23.9 \cdot 10^{-13}$ but several other components were present in concentrations ranging from 2 to 11 per cent of the analytical value. The increase in the proportion of protein with higher sedimentation constants with increase in antibody content is readily seen in the comparison of Experiments 20 and 21 in Fig. 3c.

Experiment 22. Type I Pneumococcus Anticarbohydrate Dissociated by Barium Hydroxide.—The specific precipitate used for dissociation (solution 79 E, Reference 3) was obtained in part from the Felton solution used for Experiments 20 and 21, but in spite of this the sedimentation diagram was quite different from that of Experiment 21, all diagrams at comparable times showing displacement toward smaller s values. The two main components were $s_{20} = 18.7 \cdot 10^{-13}$ (46 per cent) and $s_{20} = 23.2 \cdot 10^{-13}$ (26 per cent), while a component of $s_{20} = 9.1 \cdot 10^{-13}$ was responsible for 17 per cent (together with a $13 \cdot 10^{-13}$ component). It is probable that even the brief treatment with barium hydroxide in the cold (3) caused appreciable dissociation of the antibody protein. In accord with this and with the precipitability of 96 per cent of the protein by S I, both the top and bottom portions of the cell contents reacted with S I, the bottom part yielding the heavier precipitate. The centrifuge was stopped just after the 18 component had reached the bottom of the cell.

⁸ This serum was obtained through the courtesy of Dr. William H. Park of the New York City Department of Health Laboratories.

Experiment 23. Type I Antipneumococcus Horse Serum.—This experiment was run on a highly potent Type I antipneumococcus serum,⁹ assaying 2.8 mg. of Type I anticarbohydrate N and over 1000 mouse protective units per ml. After dialysis against 0.2 M NaCl solution the serum was diluted to about 1 per cent of protein with the salt solution. The sedimentation diagram showed the normal peaks due to albumin (33 per cent) and globulin (24 per cent) and several rather indistinct peaks, the main one having $s_{20} = 17 \cdot 10^{-13}$ (12 per cent). Between the normal globulin peak and a small peak with $s_{20} = 11 \cdot 10^{-13}$ (2 per cent) the diagram indicated about 10 per cent of inhomogeneous material (Fig. 3d).

Experiment 24. Type I Antipneumococcus Horse Serum Deprived of Anticarbohydrate.—2.0 ml. of the above serum were precipitated in the cold with 2.4 ml. of a 1:2000 solution of S I, removing practically all anticarbohydrate. After standing overnight the mixture was centrifuged in a cold room. This serum also was run at a concentration of about 1 per cent protein. The sedimentation diagram showed a considerable reduction in the relative concentration of the faster sedimenting molecules (Fig. 3d).

Experiments 25 and 26. Type I Antipneumococcus Horse Serum without Preservative, and the Same Deprived of Anticarbohydrate.—This serum was obtained from a later bleeding of the Stockholm horse and the antibody (0.65 mg. N per ml.) was removed from one portion with S I as described above. Solutions containing about 1 per cent of protein were used. The sedimentation diagrams of even this weak serum indicated a slight decrease in the concentration of the heavier molecules after removal of the antibody. There was also possibly a slight decrease in the albumin peak.

The above experiments were, in general, carried out with a protein concentration of about 1 per cent because of lack of time for runs at several dilutions. This concentration was chosen because it could be expected (15) that changes in the serum would be more marked in dilute solution than in the concentrated serum, in which the effect described in References 10 and 15 might entirely mask the changes. Several experiments were run, however, in which the serum was not diluted, but merely dialyzed against 0.2 M NaCl solution.

Experiments 27 and 28. Undiluted Type I Antipneumococcus Horse Serum, without Preservative, and the Same Deprived of Anticarbohydrate.—The serum was the same as used in Experiments 25 and 26, but undiluted. The sedimentation

⁹ The serum was supplied by Dr. Percival Hartley of The National Institute for Medical Research, Hampstead, London, to whom we are also most grateful for laboratory facilities and his personal aid.

diagrams were almost identical, regardless of the presence or absence of antibody, and gave the same values for the concentration of the three distinct peaks present, whereas the same sera showed distinct differences in dilute solution. Most of the faster sedimenting molecules had disappeared from the diagrams of the concentrated sera, the globulin peak had greatly diminished, and that due to the albumin had increased markedly (*cf.* 10) (see Fig. 3*c*, in which a comparison is given of the diluted and undiluted antiserum).

Experiment 29. Same Serum as in Experiment 23, but Undiluted.—The result was very much as above—an increase in the apparent albumin concentration and a decrease in that of the faster sedimenting molecules. It has, however, been amply shown that the amount of antibody is the same at all dilutions (2*d*).

DISCUSSION

In the present study a comparison has been made in the ultracentrifuge of normal and anti-egg albumin sera of known protein and antibody content from the same rabbit, normal and immune rabbit globulin, Felton solutions from normal and Type I antipneumococcus horse sera, and horse and rabbit pneumococcus anticarbohydrate of which up to 98 per cent of the protein present could be accounted for as antibody.

Let us first consider the rabbit material. It is evident from the data summarized in Table I and Fig. 2 that in moderately dilute solution the same sedimentation constant is obtained for the principal globulin component of normal rabbit serum, of isolated normal globulin, of anti-egg albumin serum, and of the corresponding isolated immune globulin. In both of the last up to about 50 per cent of the globulin present was specifically precipitable by egg albumin. Moreover, specimens of Type III pneumococcus anticarbohydrate (Experiments 7 and 8) produced in the rabbit, in which approximately 90 to 95 per cent of the protein present was accounted for as antibody, not only showed the same sedimentation constant but were monodisperse (Fig. 2*c*), indicating that the constant observed was actually that of the antibody. For all of this material the sedimentation constants varied from 6.6 to $7.4 \cdot 10^{-13}$, with an average of $7.1 \cdot 10^{-13}$, a value close to that found for the principal globulin fraction of other normal mammalian sera studied in Upsala (13, 10, 20, and unpublished data). It appears, therefore, that the molecular weight, at least of the two classes of rabbit antibodies included in this study, is

very close to that of normal serum globulin, about 150,000. The general applicability of this result for rabbit antibodies is further indicated by the finding of the same sedimentation constant, $7 \cdot 10^{-13}$, by Biscoe, Herčík, and Wyckoff (5) for a preparation containing anti-azoprotein, although the proportion of antibody present was not known. Goodner, Horsfall, and Bauer (6), however, reported much antibody in large aggregates in ultrafiltration studies on Type I antipneumococcus rabbit serum.

TABLE I

Observations on Normal and Immune Rabbit Sera, Globulin, and Antibody Solutions

Experiment No.	Material used	Concentration		$S_{20} \cdot 10^{13}$ principal globulin component	$S_{20} \cdot 10^{13}$ globulin component next in amount	Dispersity
		Total N	Anti-body N			
		mg. per ml.	mg. per ml.			
1	Sterile, normal rabbit serum, no preservative	1.96		7.1	17.4	Globulin nearly monodisperse
2	Anti-Ea serum from same rabbit, no preservative	2.21	0.6	6.6	13.5	" "
3	Normal globulin, same rabbit	1.36		7.1	18.4	" "
4	Immune (anti-Ea) globulin, same rabbit	1.49	0.73	7.4	19.6	" "
5	Anti-Ea globulin, rabbit 4.37 ₂	2.90	1.05	7.4	20.7	" "
6	Anti-Ea globulin, rabbit 4.31 ₂	1.42	0.48	7.3	20.3	" "
7	Rabbit Type III pneumococcus anticarbohydrate 3.50 ₁	0.11	0.10*	7.0		
8	Same, rabbit 3.51 ₁	0.64	0.57	7.0		Homogeneous

* Analysis made 2 months previously.

On the basis of the observed sedimentation constant it would appear that antibody in the rabbit is formed either from the principal globulin component of the serum or possibly by the cells or tissues responsible for the building up of the principal component of normal serum globulin. Thus the finding of a molecular weight characteristic of normal globulin might be taken as evidence in favor of the theory

of antibody formation put forward by Breinl and Haurowitz (21) and by Mudd (22), a theory, which, although widely accepted, has up to the present lacked an experimental basis.

TABLE II
Observations on Horse Sera, Concentrates, and Antibody Solutions

Experiment No.	Material used	Concentration		$S_{90} \cdot 10^{10}$ principal component	$S_{90} \cdot 10^{10}$ additional components in largest amounts	Dispersity
		Total N	Precipitin N			
		mg. per ml.	mg. per ml.			
16	Normal horse globulin (13) Felton solution, normal horse serum	1.07		7.1 8.1	10.5, 18.8 12.9, 18.5	Polydisperse "
17	Felton solution, Type I antipneumococcus serum containing no preservative	1.14	0.33	18.3	6.8, 24.2	"
18	Dissociated antibody from same serum	0.92	0.47	18.5		Homogeneous
19	Dissociated antibody from similar serum	1.08	0.66	18.2		"
20	Felton solution, Type I antipneumococcus serum preserved with phenol-ether	1.27	0.63*	9.1	17.1, 27.3	Polydisperse
21	Dissociated antibody from above Felton solution	0.98	0.85*	23.9	19.2, 11.1, 30.6	"
22	Antibody dissociated by Ba(OH) ₂ method	0.55	0.53*	18.7	9.1, 13.3, 23.2	"
Globulins						
23	Sterile Type I antipneumococcus serum	1.47	0.33	7.6	17.0, 10.9	"
24	Same serum without anticarbohydrate	1.57		7.3	18.4†	"
25	Sterile Type I antipneumococcus serum containing no preservative	1.46	0.09	7.0	18.6	"
26	Same, without anticarbohydrate	1.70		7.5	20.6	"

* From analyses made 2 to 3 months previously. The solutions had undergone no visible change.

† Experiments 24 and 26 showed smaller amounts of the heavier components than did the whole sera. See Fig. 3 d.

As regards antibodies produced by the horse, a more complicated state of affairs is indicated. From the data summarized in Table II and Fig. 3 the following will be noted. The principal component of the Felton solution obtained from fresh, normal horse serum has a sedimentation constant somewhat higher than that of normal serum globulin. On the other hand, there is relatively less material with this sedimentation constant in the Felton solutions prepared from Type I antipneumococcus horse sera and it is accompanied by significant amounts of a component of $s_{20} = 18.1 \cdot 10^{-13}$ and heavier molecules as well. Likewise most of the protein in Type I pneumococcus antiscarbohydrate solutions, prepared according to Reference 3 and containing roughly from 50 to 98 per cent of the protein in the form of antibody, showed sedimentation constants of $s_{20} =$ about $18 \cdot 10^{-13}$, except for the material in a salt-dissociated specimen obtained from phenol-ether-treated antiserum, which will be discussed below.

Two preparations of the antibody fraction dissociated by strong salt (3) from the specific precipitate from Type I antipneumococcus horse serum containing no preservative showed homogeneous sedimentation, s being 18.2 and $18.5 \cdot 10^{-13}$. These observations are in accord with conclusions based on diffusion measurements (2*d*),¹ ultrafiltration experiments on a Type I antipneumococcus horse serum by Elford, Grabar, and Fischer (4), and ultracentrifugal studies on Felton concentrates reported in a preliminary note by Biscoe, Herčik, and Wyckoff (5). They do not, however, account for the enormous aggregates postulated by Goodner, Horsfall, and Bauer (6) as a result of the ultrafiltration of Type I antipneumococcus horse serum. All reports indicate, however, that pneumococcus I antiscarbohydrate in the horse consists of protein molecules of high molecular weight.

In the horse antibody solutions studied thus far in the work the antibody had been precipitated during its preparation, either with specific polysaccharide or by pouring into water. While the former procedure had not increased the molecular size of rabbit antibody, it seemed possible that precipitation of antibody formed by the horse might result in an aggregation which would not necessarily be entirely reversible, and that this might account for the high sedimentation

constant observed. Two different Type I antipneumococcus horse sera were accordingly diluted and run in the ultracentrifuge before and after removal of the anticarbohydrate. Both showed a notable reduction chiefly in the more rapidly sedimenting molecules, indicating again that the antibody occurred in these fractions of high molecular weight.

Eliminating the values found in Experiments 20 and 21 as out of line, the sedimentation constant of the principal component of the anticarbohydrate-containing solutions varies from 18.2 to $18.7 \cdot 10^{-13}$, with an average of $18.4 \cdot 10^{-13}$. The sedimentation constant alone does not define the molecular weight of the antibody, since sedimentation depends not only on the molecular weight of the particle but also on its shape, and this is at present unknown. By comparison with the molecular weights of molecules having sedimentation constants at about $18.4 \cdot 10^{-13}$, one could guess at a molecular weight of about one-half million, or 3 to 4 times that of normal globulin. It should be noted that a small component of approximately this magnitude is characteristic of the globulin of most normal sera so far investigated, including the rabbit sera shown in Fig. 2. It will be seen from this figure that the amount of this component is not increased in immune rabbit sera or globulin, and, indeed, is not present in appreciable amount in highly purified anticarbohydrate produced by the rabbit. There would thus appear to be a fundamental difference in the mechanism of formation at least of pneumococcus anticarbohydrate in the horse and in the rabbit, in that this antibody arises in the horse by development of an otherwise minor globulin fraction which the rabbit does not use either for the production of pneumococcus anticarbohydrate or for other antibodies (see, however (6)).

Since there are many differences, such as water precipitability, for example, between pneumococcus anticarbohydrate and other antibodies such as diphtheria antitoxin produced by the horse, it is possible that different antigens produce different stimuli in this animal, resulting in a more varied response than in the rabbit. Information along these lines is being sought in this laboratory.

It would be reasonable and tempting to assume that the high molecular weight of pneumococcus anticarbohydrate in the horse is the cause of many of the observed chemical and physiological differences

in this antibody as produced in the horse and in the rabbit. Thus even slight degradation of the specific polysaccharide is sufficient to decrease the amount of rabbit antibody it precipitates (19) without affecting the quantity of homologous horse antibody thrown down, while by more severe degradation it is possible to obtain polysaccharide preparations which do not precipitate rabbit antibody but still throw down that produced by the horse (23). Possibly even the failure of horse anticarbohydrate to sensitize guinea pigs passively to the homologous polysaccharide or to fix complement with it might be ascribed in part to the large size of the horse anticarbohydrate molecule. If these surmises have any basis they would also raise the question as to whether or not the horse is the best animal from which to draw serum for use in antipneumococcus therapy.¹⁰ Experiments have therefore been undertaken along these lines.

It will be observed from Table II and Fig. 3 that the sedimentation behavior of antibody solutions prepared from sera which had been preserved with phenol and ether was more complex than that of antibody solutions obtained from sterile, unpreserved sera. This is in accordance with an earlier finding of McFarlane (20) and is possibly due to the alteration or denaturation of a portion of the serum proteins by the preservative. However, the phenol-ether-treated serum contained more antibody than did the one yielding the simpler solutions and this may also have influenced the results. The use of 0.01 per cent merthiolate¹¹ did not seem to affect the sedimentation picture.

Brief treatment of the antibody with alkali (Experiment 22) resulted in a shift toward more slowly sedimenting molecules. Even the smallest of these were specifically precipitable, as shown both by analysis of the entire solution and by tests on the upper and lower portions of the cell contents after centrifugation. On the other hand, only the lower portion of the cell contents reacted for antibody in the case of a Felton solution (Experiment 20), providing

¹⁰ Since the completion of this work, this question has been raised by Horsfall, Goodner, and MacLeod (*Science*, 1936, 84, 579).

¹¹ The merthiolate used in this investigation was presented by the manufacturers, Eli Lilly and Company, Indianapolis.

additional evidence for the occurrence of the antibody in the more rapidly sedimenting fractions under ordinary conditions.

Antibody solutions of a high degree of purity may thus vary from the monodisperse to the polydisperse in the ultracentrifuge, depending, at least in part, upon the treatment to which the protein has previously been subjected. The property of precipitability by means of the specific polysaccharide is not affected within the range studied and thus extends over considerable differences in molecular size. Nevertheless, for studies on the dispersity of antibodies it would appear desirable to avoid the use of preservatives whenever possible. Furthermore, even in cases in which the presence or absence of antibody is shown by distinct changes in the sedimentation diagrams of diluted sera (Experiments 25 and 26), the diagram produced with the undiluted antiserum may be impossible to distinguish from that obtained with the same serum deprived of its antibody and centrifuged at the same high total protein concentration (Experiments 27 and 28). Thus antibodies, like many other proteins ((15) and page 400), appear to be dissociated into smaller molecules in concentrated protein solutions, and the sedimentation diagram in the undiluted serum becomes more obscure. It therefore seems preferable at present to study the changes caused by immunization in the sedimentation diagrams of dilute sera and antibody solutions, especially since it has been shown that the antibody content of a serum does not depend on the dilution at which the analysis is carried out (2*d*).

Data are also given in Table III and Fig. 2 on the ultracentrifugal behavior of egg albumin-anti-egg albumin precipitates dissolved in an excess of egg albumin. These experiments were made not only because they afforded a means of obtaining solutions in which anti-egg albumin comprised more than 50 per cent of the protein present (Experiments 9, 12, 15), but also because of their theoretical implications. Thus the inhibition zone of the precipitin reaction may be considered due to peptization of the specific precipitate by excess of antigen, or due to the formation of soluble compounds between antibody and relatively much antigen (2*a*, 2*d*, 16, 17). It was hoped that the behavior of the dissolved precipitates in the ultracentrifuge would throw some light on whether the colloid chemical or the classical chemical theory applied.

Actually, interpretation of the results was rendered somewhat difficult, not only by the complex sedimentation diagrams of occasional solutions, but also by the shift toward lower sedimentation constants caused by a larger excess of egg albumin. If, however, the results are considered due to the effect recently reported (15)

TABLE III
Observations on Dissolved Egg Albumin-Anti-Egg Albumin Precipitates

Experiment No.	Material used	Analytical concentrations		Concentrations from sedimentation diagrams		Mean $S_{20} \cdot 10^{13}$ principal globulin component	$S_{20} \cdot 10^{13}$ globulin component next in amount	Dispersity
		Total N	Anti-body N	Ea	Ea-anti-body*			
		mg. per ml.	mg. per ml.	gm. per 100 ml.	gm. per 100 ml.			
9	Ea-Anti-Ea precipitate dissolved in Ea	1.53	0.93	ca. 0.3 to 0.4	0.4	12.8	26.4	Polydisperse
10	Same, large excess Ea	1.82	0.65	0.54	0.6	9.0	7.2, 16.2, 19.1	"
11	First half precipitate, large excess Ea	3.4	1.4	0.73	1.00	9.7	12.3	"
12	Second half precipitate, less Ea	1.9	1.2	0.31	0.75	12.1	13.3	"
13	Solution 11, 3 days later	3.4	1.4	(0.7)†	1.25	9.9		"
14	First half precipitate, excess Ea	1.49		0.29	0.5	9.6		"
15	Second half precipitate, slight excess Ea	1.29		0.11	0.5	14.1	18.1, 10.7, 11.6	Several apparently homogeneous peaks

* Total concentration of all components with s above $7 \cdot 10^{-13}$.

† Uncertain.

they seem intelligible. Thus in Experiment 15 (Fig. 2 e), with only a very small excess of egg albumin, there are a number of almost homogeneous complexes in the solution, consisting apparently of relatively simple combinations between the antibody and the antigen. With increasing egg albumin concentration, as in the other experiments, these complexes would be in part dissociated, forming

increasing numbers of smaller molecules as the egg albumin concentration increases. The mean sedimentation constant would therefore decrease at the same time, as found experimentally. Since all sedimentation constants found, however, were larger than that for antibody alone, the results are consistent with the theory that soluble chemical compounds of antigen and antibody are actually formed. These findings are also consistent with the theory that specific precipitates are built up by the interaction of multivalent antigen (or hapten) with multivalent antibody to form large aggregates (2*d*, 2*e*; *cf.* also 16, 17), since the molecular species found after the precipitate has been dissolved with a minimum antigen excess are larger than those in solutions containing much antigen. The Svedberg ultracentrifuge has thus furnished new insight into the complex chemical reactions involved in the inhibition zone of the precipitin reaction. The extension of its use to other immune systems in which precipitation is not involved would appear promising.

Finally the present studies, by making available approximate molecular weights for certain antibodies, have made it possible to calculate actual molecular formulas for some of the compounds formed in specific precipitation. This phase of the work will be taken up in a separate communication.

We wish again to express gratitude to Professor The Svedberg for his kind extension of the hospitality of his Institute and for his freely given counsel, and to Dr. Arne Tiselius and the other members of Professor Svedberg's staff for their assistance and for many courtesies.

SUMMARY

1. Highly purified rabbit Type III pneumococcus anticarbohydrate proved to be homogeneous in the ultracentrifuge and its sedimentation constant, $7.0 \cdot 10^{-13}$, did not differ from that of the principal component of normal rabbit globulin or of immune rabbit globulin containing up to 50 per cent of anti-egg albumin. The molecular weight of antibody in the rabbit is therefore probably very close to that of the principal normal globulin component, namely, 150,000.

2. Highly purified horse Type I pneumococcus anticarbohydrate, on the other hand, was only homogeneous in the ultracentrifuge when prepared from sera stored without preservative. Its sedimen-

tation constant, $18.4 \cdot 10^{-13}$, coincided with that of the principal globulin component in most of the Felton solutions and purified antibody solutions studied. The molecular weight of pneumococcus anticarbohydrate in the horse is probably three to four times that of the principal normal globulin component.

3. The significance of the differences between pneumococcus anticarbohydrate formed in the rabbit and in the horse is discussed.

4. Results are given of ultracentrifuge studies on the molecular species in solutions of egg albumin-anti-egg albumin specific precipitates dissolved in excess egg albumin. The implications of the results are discussed.

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ANTIDIURETIC PITUITARY SUBSTANCE IN BLOOD, WITH SPECIAL REFERENCE TO THE TOXEMIA OF PREGNANCY

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Since the work of Anselmino and Hoffmann (1) concerning a possible relationship between the existence of pituitary antidiuretic substance in blood and the clinical symptoms of the toxemias of pregnancy, this question has attracted much attention. The experimental data in the literature are rather scant and confusing, and it was with the hope of throwing further light upon the subject that the experiments described herein were undertaken.

At the outset, it may be stated that the great difficulty in respect to this problem is the lack of a suitable method for the extraction and concentration of the hormone from blood. Indeed, it is still doubtful whether or not antidiuretic pituitary substance is a normal physiological blood constituent. Naturally, it was necessary to test out this point first.

The antidiuretic substance described by Anselmino and associates was found in ultrafiltrates obtained from plasma of toxemic patients, and has not been adequately characterized as the pituitary hormone. Theobald (2), de Wesselow and Griffiths (3), Marx and Schneider (4), and Levitt (5), have all tested this procedure, and were led to the conclusion that ultrafiltration of plasma through the acetic collodion filter employed does not remove quantitatively posterior pituitary hormone previously added to the plasma. Apparently, the recovery of activity in material thus treated is extremely variable according to the investigator. Furthermore, Byrom and Wilson (6) and Hurwitz and Bullock (7) failed completely to obtain any antidiuretic activity from such ultrafiltrates. It may be concluded then that this procedure is too uncertain for the satisfactory quantitative examination of the problem in question.

Recently, Marx and Schneider (4) described a method for the extraction and concentration of an antidiuretic substance from blood, using methyl or ethyl

alcohol as the solvent. This substance was found to be a constituent of normal blood and, in comparison with the effects produced by pitressin, an extract prepared from 100 cc. of blood showed approximately 0.005 to 0.01 unit of activity. None of several samples of pathological blood studied by Marx and Schneider showed any alteration in this concentration. Hence, it was concluded that this was a physiological blood constituent and not altered in any disease condition. No cases of pregnancy toxemia were studied by these workers. With regards to the solvent action of alcohol for the active substance in question, it may be added that 96 per cent ethyl alcohol was also found by Stehle (8) to be the best solvent for both the pressor and oxytocic principles during their purification and separation from the posterior lobes of the gland itself.

As it is now generally agreed that the antidiuretic activity of posterior lobe extracts is due to the so called pressor principle, the method employed for its extraction should be equally applicable when tested by the pressor assay method. This has been the case. No attempt has been made, however, to detect pressor active quantities of this material either in normal or diseased blood, since it has been clearly shown by Stehle (9) and others that it requires about 200 times larger amounts of this substance to effect a minimal blood pressure rise than to produce a very definite antidiuretic action. In fact, the effects of pituitary extracts upon the blood pressure of the normal unanesthetized animal are so variable (Melville (10)), and such large quantities of active material are required, that it is questionable whether a concentration high enough to affect the blood pressure could exist in the blood stream without exerting very pronounced effects upon the urine secretion. Moreover, according to recent work of Page (11), alcoholic blood extracts may show pressor effects undoubtedly due to some other constituent than the pituitary hormone.

The object of this paper is primarily to describe a workable and accurate method for the demonstration of antidiuretic pituitary active substance in dog and human blood, and to describe some preliminary findings in connection with the problem stated in the first paragraph.

Method of Extraction of Antidiuretic Substance

The method which was finally adopted for the extraction and concentration of the antidiuretic substance from blood is briefly as follows:

The freshly drawn blood is shaken with heparin in order to prevent clotting. A measured volume is then placed in a Florence flask and mixed thoroughly with

nine volumes of 80 per cent ethyl alcohol, which was found to give the best results under these conditions. The mixture is then acidified to Congo red paper by the gradual addition of normal sulfuric acid. After being shaken for several minutes, the flask is stoppered and left at room temperature (22°C.) for 3 to 4 hours, with repeated shaking. The solution is then filtered with a Buchner filter and the residue washed twice with 80 per cent ethyl alcohol. The total filtrate is then concentrated on a water bath kept at a temperature below 70°C., under reduced pressure, using a water pump. When about one-fifth of the original volume is left, the concentrate is cooled and either filtered or centrifuged. The clear brownish solution obtained is then evaporated to dryness at room temperature by being placed overnight in front of an electric fan. When the quantity of blood employed does not exceed 20 cc., this residue is quite small, and it suffices only to dissolve it in 1 or 2 cc. of distilled water and neutralize with normal sodium carbonate just prior to injection. When the sample of blood employed is larger, it is necessary to extract the residue a second time, using 20 to 25 cc. of 80 per cent alcohol, then chase the alcohol and concentrate as before, centrifuging and finally evaporating to dryness at room temperature. After this second extraction, the residue obtained is treated as for the smaller samples.

The active residue is quite stable in the dried state, and, after evaporation to dryness in acid medium, samples kept in the laboratory for as long as 7 days showed no appreciable loss of activity. The final solution obtained was always slightly discolored, and all attempts to obtain a water-clear extract with complete recovery of active substance were unsuccessful.

Several other methods of extraction have been tested, but in each instance active material added to blood was either completely lost or such a small fraction recovered as to render such methods useless. Some interesting results obtained with modifications of the above described procedure will be referred to later, especially in reference to the necessity for acidification during the extraction.

Tests of Activity of Extracts

The extracts were usually tested for their antidiuretic potency, using unanesthetized bladder fistula dogs. In the preparation of these animals ether anesthesia was employed and the bladder exposed through a low midline incision. As much as possible of the anterior wall of the bladder was excised and the margins of the residual tissue suitably sutured to those of the incised abdominal wall. The urine could then be observed freely discharging from the ureteral orifices. After about 10 days the wound is healed and a permanent fistula is established.

In each experiment 300 cc. of water were given by stomach tube at 10 a.m.

and again at 2 p.m. After the second administration the animal was placed comfortably in the prone position on a table in such a manner that the fistular opening was directly over the top of a suitably arranged funnel into which the urine discharged and was collected and measured in a graduated cylinder. The animals rapidly became conditioned to the procedure and would remain quietly on the table without any restraint during the whole course of the experiment. In most of the experiments the antidiuretic effect obtained from the extract was compared with that obtained from the injection of a known dose of a pressor pituitary preparation, for which purpose either pitressin or our own laboratory preparation, postlobin-V (8), was employed. All injections were made intravenously, and in each instance the rate of urine flow at the time of injection was allowed to attain approximately 2 cc. per minute, in order to be sure that a good diuresis was developing.

The antidiuretic effects observed under such conditions are fairly constant for a given dose of the extract in the same animal, and, as a matter of fact, when the animal is well hydrated at the beginning of the experiment, three or four injections of small dosages of the extract can be carried out in a single experiment with quite uniform results.

In all the graphs shown, the ordinates represent cubic centimeters of urine per minute and the abscissae the time in minutes following the second administration of water.

In a few experiments, the blood pressure method of assay was employed in order to check the recovery of much larger quantities of pressor pituitary extract added to blood. In these, the chloretonized dog was used and the blood pressure recorded directly from a carotid artery.

All injections were made intravenously and the pressor effects obtained compared with standards of known potency.

EXPERIMENTAL

Recovery of Antidiuretic or Pressor Pituitary Substance Added to Blood in Vitro and in Vivo.—In Figs. 1 and 2 are shown some examples of antidiuretic effects obtained with blood extracts from normal dog blood to which had been added pitressin or postlobin-V *in vitro*. In Fig. 1 the extract injected at A was prepared from 5 cc. of dog blood to which was added 0.01 unit of postlobin-V, and the response observed was quite similar to that following the injection of an equal amount of the original postlobin-V preparation, as shown at B.

Similar results are shown in Fig. 2. Here, at A, it is seen that an injection of an extract made from 10 cc. of normal dog blood exerted no antidiuretic effect. At B, 0.005 unit of pitressin was given and the response is again almost identical with the effects obtained after

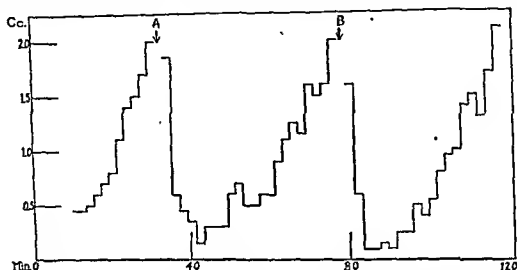


FIG. 1. In this and the succeeding graphs, the ordinates represent cubic centimeters of urine secreted per minute, and the abscissae time in minutes following an administration of 300 cc. of water by stomach tube (see text). A, extract from 5 cc. dog blood with 0.01 unit postlobin-V added. B, 0.01 unit postlobin-V.

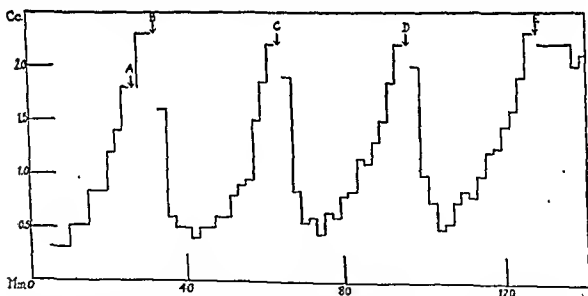


FIG. 2. A, extract from 10 cc. dog blood. B, 0.005 unit pitressin. C, extract from 10 cc. dog blood with 0.005 unit of pitressin added. D, same as C. E, control saline injection.

the injections indicated at C and D, where blood extracts made from 10 cc. samples of blood to which had previously been added 0.005 unit of pitressin were given. In the case of D, however, the mixture of the pituitary extract and the blood was allowed to stand at room

temperature for 4 hours prior to the extraction. This delay in extraction does not therefore, apparently, interfere with the recovery of the active material. As a matter of fact, a similar extract made from blood which had been mixed with pitressin and allowed to stand for 24 hours before extraction showed no significant difference. Finally, at E a control saline injection produced no effect.

It has further been possible to obtain definite antidiuretic effects when quantities as small as 0.0025 and 0.001 unit of pressor pituitary hormone were added to samples of as much as 25 cc. of normal dog blood, although the recovery was not as complete as when larger quantities were used. This method would appear therefore capable of demonstrating quantitatively the presence of even such minute amounts of antidiuretic active substance were these present in the blood stream.

A number of experiments were also undertaken in order to test the above described procedures upon samples of blood mixed with pitressin or postlobin-V *in vivo*. For this purpose dogs were injected intravenously with 1 unit per kilo of the extract, and samples of blood taken after 5 and 30 minutes, respectively. These samples were mixed with heparin and the antidiuretic effects produced by 1 cc. quantities when injected directly into the vein of the bladder fistula animal, compared with that produced under identical conditions but after submitting the sample to the extraction process described above. In Fig. 3 are shown some examples of the results thus obtained. The results with the samples of unextracted blood (continuous line) are superimposed upon those obtained with the samples of extracted blood (broken line). Thus at A, 1 cc. of heparinized dog blood, withdrawn 5 minutes after the pituitary injection, was administered, and at A₁, the extract prepared from an equal quantity. Again, at B, 1 cc. of blood taken 30 minutes after pituitary and at B₁, the extract prepared from an equal quantity, were respectively injected. The close agreement between the two sets of results is quite striking, and leaves little doubt that the method employed is equally capable of demonstrating the presence of pituitary antidiuretic substance in the circulating blood.

At C in Fig. 3 is shown by way of comparison the effect obtained from an injection of 0.005 unit of postlobin-V in one such experiment. The quantity of active substance then in 1 cc. of the animal's blood

5 minutes after the original injection was definitely less than the equivalent of 0.005 unit. Assuming a uniform distribution of the injected substance, and the total blood volume being approximately one-thirteenth of the animal's weight, the blood should have contained approximately 0.076 unit per cc. Obviously, therefore, the injected antidiuretic material disappears with extreme rapidity from the circulating blood. According to Dale (12) and Heller and Urban (13) this is largely due to a rapid urinary elimination. In one

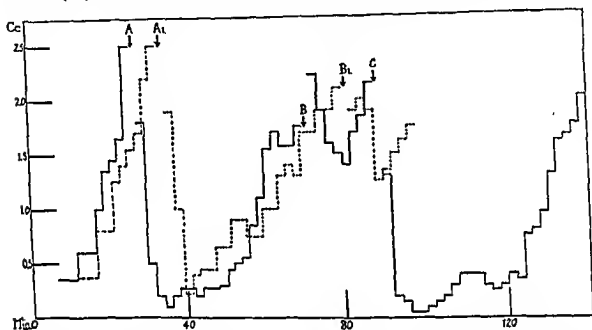


FIG. 3. Experiment 1 (continuous line graph). A, 1 cc. heparinized dog blood withdrawn 5 minutes after intravenous injection of 1 unit postlobin-V per kilo. B, 1 cc. heparinized dog blood withdrawn 30 minutes after same injection. C, 0.005 unit postlobin-V.

Experiment 2 (broken line graph). A₁, extract of 1 cc. heparinized dog blood withdrawn 5 minutes after intravenous injection of 1 unit postlobin-V per kilo. B₁, extract of 1 cc. heparinized dog blood withdrawn 30 minutes after same injection.

experiment the urine secreted from a bladder fistula dog for a period of 10 minutes after a similar pituitary injection, when extracted showed definite antidiuretic activity, so that some of the loss is undoubtedly through this channel.

In order to verify further the accuracy of the above procedure in extracting specifically the pituitary hormone, some tests were carried out using much larger quantities of pitressin or postlobin-V added to blood, extracting by the method described above, but assaying the

extracts obtained on the blood pressure rather than upon the urine secretion. It was also hoped in the course of such experiments to demonstrate further the relatively high concentrations of the hormone which would have to exist in the blood in order to render such a method of its detection at all feasible. Some results are shown in Figs. 4 and 5.

In Fig. 4, at A, 0.5 unit of postlobin-V was injected. At B, the extract obtained from 5 cc. of normal dog blood was given as a control. At C is shown the effect produced by an extract obtained from 5 cc. of normal dog blood to which had been added 0.5 unit of postlobin-V. A and C agree quite well. Similarly, at G and H are shown, respectively, effects obtained from the injection of an extract prepared from 5 cc. of blood to which had been added 1 unit of postlobin-V (G), and from that of 1 unit of postlobin-V alone (H). The two effects are again almost identical. When, however, a sample of blood was mixed with 1 unit of postlobin-V as above, and extracted without acidifying with sulfuric acid, the active material was either completely destroyed or so reduced as to be not detectable upon the blood pressure (D). Alcoholic extraction therefore, without acidification, such as has been employed by Marx and Schneider (4), cannot quantitatively remove posterior lobe active substance added to blood.

A convenient method of treating blood before extracting with acid-alcohol is to dry and pulverize by mixing with anhydrous sodium sulfate, and for some time this procedure seemed very promising, as in this instance the extract obtained is generally water-clear. In extracts thus prepared it was found, however, that only approximately 50 per cent of activity could be recovered. Thus at F in the same figure the effect observed was only half of that shown at G, although the quantity of postlobin-V which was added to the sample thus treated was equal in both instances.

The results shown in Fig. 5 are quite similar and need little comment. It should be stated, however, that 10 cc. samples of blood were employed throughout this experiment. At D, also, is shown the exceedingly poor recovery of active material, even when as much as 5 units of postlobin-V were added to the sample of blood and the extraction carried out without the addition of acid. In this instance the response is less than that shown at A, after an injection of only

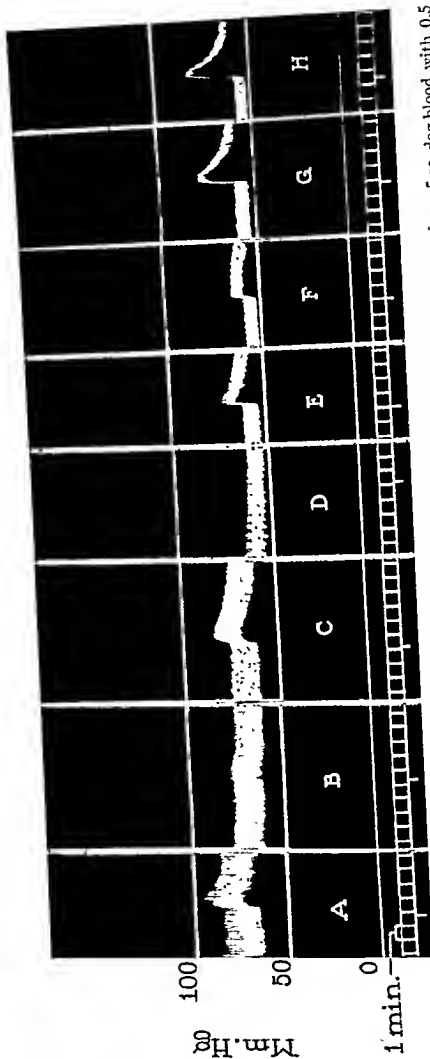


FIG. 4. Blood pressure tracing. A, 0.5 unit postlobin-V. B, extract from 5 cc. dog blood. C, extract from 5 cc. dog blood with 0.5 unit postlobin-V added. D, non-acidified extract from 5 cc. dog blood with 1 unit postlobin-V added. E, same as A. F, extract from 5 cc. dog blood with 1 unit postlobin-V added, but previously dried with anhydrous sodium sulfate. G, 1 unit postlobin-V. H, extract from 5 cc. dog blood with 1 unit postlobin-V added.

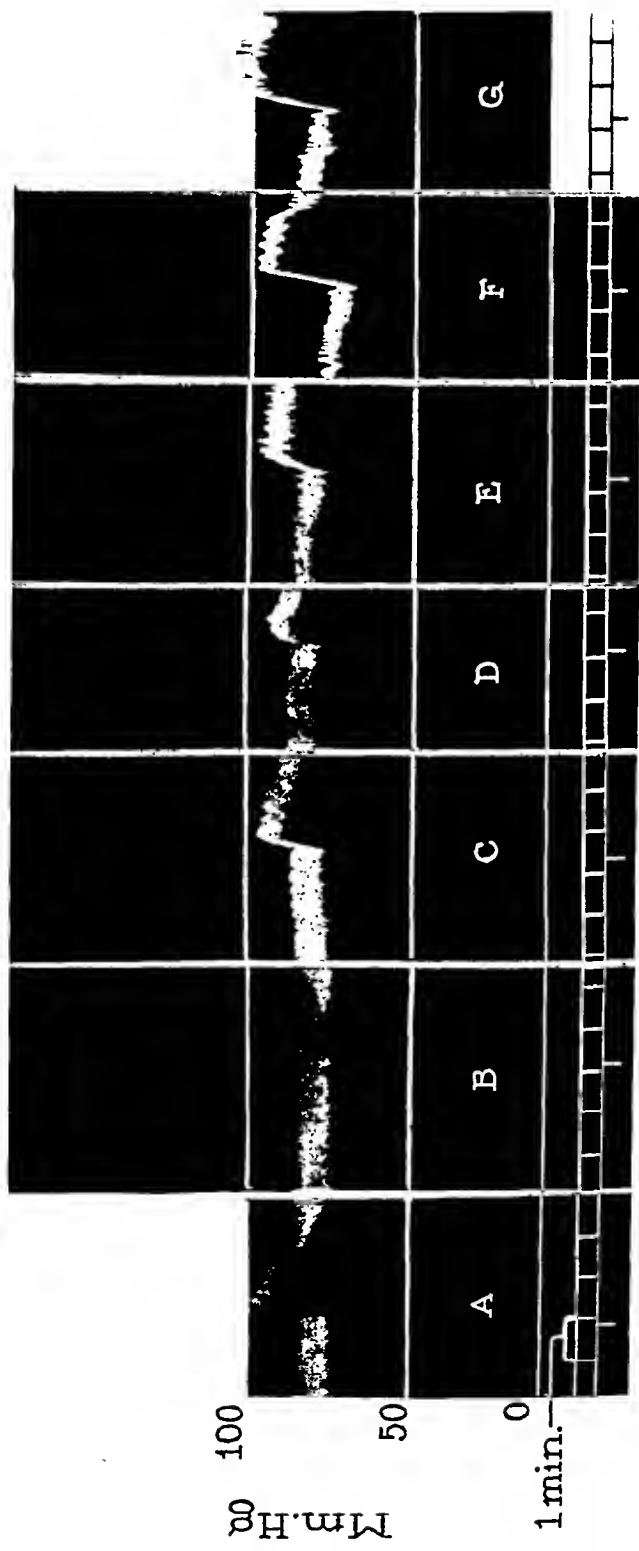


FIG. 5. Blood pressure tracing. A, 0.5 unit postlobin-V. B, extract from 10 cc. dog blood. C, extract from 10 cc. dog blood with 0.5 unit postlobin-V added. D, non-acidified extract from 10 cc. dog blood with 5 units postlobin-V added. E, same as C. F, 1 unit postlobin-V. G, extract from 10 cc. dog blood with 1 unit postlobin-V added.

0.5 unit. These results confirm those described earlier, and leave little doubt that the method of extraction employed in the antidiuretic experiments is equally applicable and accurate on the basis of the pressor assay method. In the latter case, however, much larger quantities of active substance (at least 0.5 unit) would have to be present in the samples of blood extracted. Furthermore, the close agreement between the findings obtained with the pressor and antidiuretic methods suggest very strongly that the procedure in question extracts the posterior pituitary hormone itself and not any other hypothetical antidiuretic and pressor substance. Indeed, it is almost

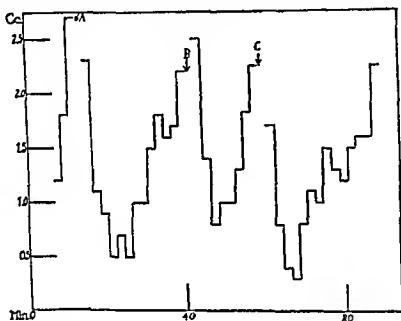


FIG. 6. A, 0.005 unit pitressin. B, extract from 50 cc. dog blood. C, extract from 50 cc. dog blood.

inconceivable that the same procedure would remove in such quantitatively accurate manner two different substances.

Antidiuretic Pituitary Substance in Dog Blood.—In Fig. 6 are shown some effects which were obtained with extracts prepared from 50 cc. samples of dog blood. After each injection, B and C, there was a definite antidiuretic effect. The quantity of active substance seems to be somewhat variable, however, and in comparison with the effect produced by an injection of 0.005 unit of pitressin (A) the blood samples contained less than this quantity per 50 cc. Smaller volumes of dog blood gave uniformly negative results. In another experiment the extract prepared from a sample of 100 cc. of blood led to a definite

inhibition of urine secretion, requiring 58 minutes to return to the pre-injection rate of secretion. It would appear therefore that dog blood contains the posterior pituitary antidiuretic substance as a

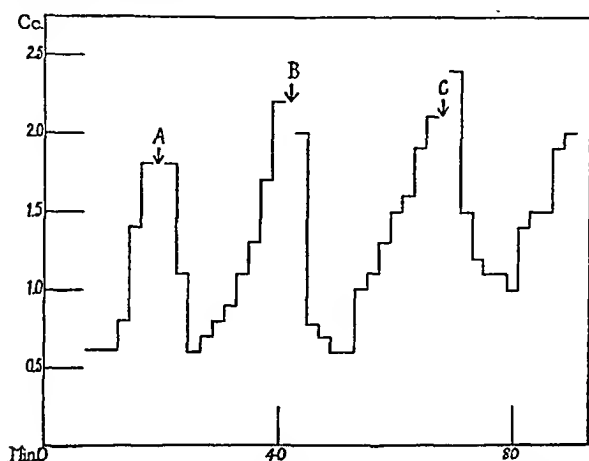


FIG. 7. A, extract from 15 cc. human blood (female non-pregnant). B, extract from 15 cc. human blood (female non-pregnant). C, extract from 15 cc. human blood (female non-pregnant).

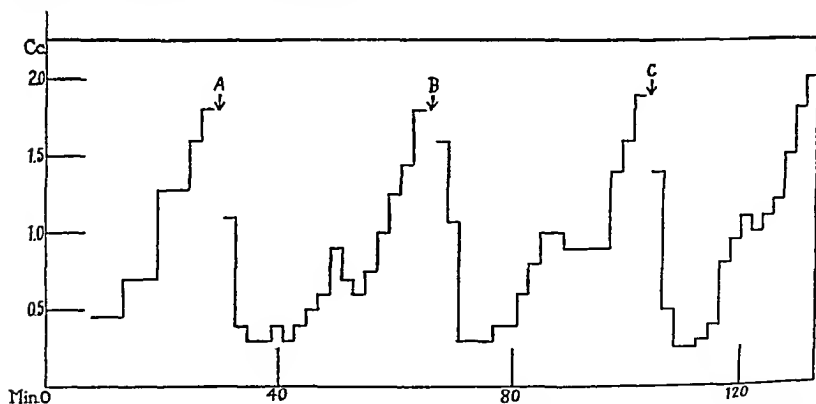


FIG. 8. A, extract from 50 cc. human blood (male). B, extract from 25 cc. human blood (male). C, 0.005 unit postlobin-V.

physiological constituent. The concentration appears, however, somewhat variable, and further investigation must be carried out in order to elucidate this point.

Antidiuretic Pituitary Substance in Human Blood.—In Figs. 7 and 8 and Table I are presented some of the results which were obtained with human blood. The results shown in Fig. 7 were obtained from injections of extracts prepared from three 15 cc. samples of blood from three non-pregnant women, and in each instance some antidiuretic response was obtained. Again in Fig. 8 are shown effects obtained in another experiment with extracts made from 50 and 25 cc., respectively, of blood obtained from two males. In this instance the effects produced are apparently more pronounced than those shown in Fig. 7 and, in comparison with that resulting from an injection of 0.005 unit

TABLE I

Case No.	Name	Age	Blood pressure	Edema	Albuminuria	Blood extract	Result
		yr.				cc.	
1	R. B.	23	128/88	Negative	Negative	8	Negative
2	C.	35	130/95	Negative	Negative	10	Negative
3	R. R.	30	155/90	Positive	Trace	8	Antidiuresis lasting 54 min.
4	R. R.	30	120/80	Negative	Negative	9	Antidiuresis lasting 28 min.
5	I. M.	28	160/90	Negative	Negative	10	Antidiuresis lasting 22 min.
6	R. L.	28	140/84	Negative	Negative	20	Antidiuresis lasting 34 min.
7	M. R.	24	162/110	Positive	Positive	15	Antidiuresis lasting 34 min.
8	M. G.	—	128/88	Negative	Negative	15	Negative
9	M. M.	—	145/90	Positive	Trace	15	Antidiuresis lasting 12 min.
10	M. W.	—	142/92	Positive	Negative	15	Antidiuresis lasting 24 min.
11	Normal pregnancy					15	Negative
12	Normal pregnancy					15	Negative

of postlobin-V, would suggest that, as in the case of the dog, normal blood contains somewhat variable amounts of the antidiuretic pituitary substance, exceeding apparently 0.005 unit per 50 cc.—more than in the case of the dog.

In Table I, the results obtained with several samples of blood taken from pregnant women are summarized. In each instance the blood was obtained during the last month of the pregnancy. The earliest symptoms of toxemia were considered as increase of blood pressure, with or without albuminuria or edema, in the previously normal pregnant woman. No cases showing advanced eclamptic convulsions were available during the period of this investigation, but this should

not detract from the possibility of detecting an augmentation of the antidiuretic substance in the blood, since, as has been stated, any amount of this substance which would be responsible for a detectable increase of blood pressure should be easily detectable by the antidiuretic test. It may be recalled in this connection that it requires experimentally more than 200 times the antidiuretic effective dose of this substance to exert a minimal effect upon blood pressure.

The results shown in the last column of the table indicate the antidiuretic effects observed with the extracts prepared, the time indicating the period required after the injection for the urine rate to return to the pre-injection level.

In general, the results were of a rather confusing nature. In view of the effects observed with 15 cc. samples of normal human blood as shown in Fig. 7, it was quite surprising to obtain entirely negative results in so many of the cases studied. Five of the twelve samples of blood of 8 to 15 cc. volumes showed no antidiuretic action. This reduction in the normal content of this substance would appear then to be related to the condition of pregnancy *per se*, but whether this is due to a true hyposecretion of the hormone in question or to an associated blood dilution cannot be stated. It is known, however, that the specific gravity of the blood is lowered in pregnancy (14). On the other hand even in the definitely toxic cases the quantity of antidiuretic substance detectable in the blood was not large. As a matter of fact in cases 5 and 7, where the vascular effects were most intense, judging from the blood pressure readings (160/90 and 162/110, respectively), there was less antidiuretic activity in the blood extracts than in much less severe cases, for example, Nos. 6, 9, and 10. In one case only, No. 3, was there any striking evidence of antidiuretic activity in the extract, but curiously enough another sample of blood taken from the same individual (No. 4), after the pregnancy was ended, showed also an exceptionally high degree of activity. All in all, therefore, the above data do not support the view that the symptoms of pregnancy toxemia (hypertension, albuminuria, edema) are in any way related to hypersecretion of the posterior pituitary gland. It is curious and should be noted, however, that in most of the toxic cases the blood did show antidiuretic active substance, which apparently is not evident in the blood in normal pregnancy. We are at a

loss to explain the phenomenon, but since as stated above the kidneys are important channels for the elimination of this substance, it is conceivable that there may be some interference with this process in the toxemic patient, hence the retention. This, however, is purely hypothetical.

SUMMARY

A method is described for the quantitative extraction of posterior pituitary antidiuretic substance from blood with which it has been mixed *in vitro* and *in vivo* for experimental purposes.

With this procedure, it is found that a similarly extractable active substance may be detected as a normal constituent of dog and human blood.

The data obtained from the blood of normal pregnancies and several cases of early toxemia, do not indicate any causal relationship between the presence of this substance in the circulating blood and the early symptoms (hypertension, edema, albuminuria) of the toxemia of pregnancy.

We are greatly indebted to Dr. J. S. Henry of the staff of the Royal Victoria-Montreal Maternity Pavilion, for the samples of blood and the clinical data used in this investigation.

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BLOOD PLASMA PROTEIN REGENERATION AS INFLUENCED BY INFECTION, DIGESTIVE DISTURBANCES, THYROID, AND FOOD PROTEINS

A DEFICIENCY STATE RELATED TO PROTEIN DEPLETION*

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Our belief that plasma proteins participate actively in the complex internal protein metabolism of the body should be adequate reason for our continued interest in blood plasma protein regeneration. It appears that the plasma can contribute protein readily to body tissues or body stores, but only in small amounts and with difficulty can the body contribute protein to the blood plasma—for example in fasting. The term "dynamic equilibrium" has been used to express the ebb and flow between plasma, organ, and tissue proteins. When reserve stores are exhausted the blood plasma production depends almost wholly upon food factors coming into the body from the intestine (16, 13, 9).

Reserve stores under ordinary conditions are adequate to tide the body over any emergency call for new formed plasma proteins. A study of the reserve stores is in progress and it is probable that the stores of materials from which plasma proteins can be fabricated are distinct from the stores of hemoglobin building material.

Infection can inhibit the formation of hemoglobin in anemia (17) and in like fashion it can inhibit the formation of plasma protein (Tables 3 and 3-a and Chart A below) during plasmapheresis. The mechanism of this interesting reaction is discussed below.

* We are indebted to Eli Lilly and Company for valuable materials used in these experiments.

A *deficiency state* which may result fatally can be produced in these dogs by long continued plasma depletion combined with low protein intake even when the accessory diet factors, salts and vitamins, are adequately represented in the diet. It is in a sense an exhaustion of the protein stores, perhaps an injury of the essential intracellular protein matrix of the body cells and a disturbance of the protein-forming mechanism. At any rate, the A/G ratio falls, resistance to infection is greatly reduced, and the output of new plasma protein drops to very low levels.

The method of these experiments is simply stated, although not always simply executed. By daily bleeding and return of washed red cells suspended in a modified Locke's solution (plasmapheresis) the normal plasma protein level in dogs (5 to 7 per cent) is reduced to about 4 per cent. Here it is maintained fairly constantly over long periods of time and presumably the hypoproteinemia acts as a strong stimulus for the regeneration of plasma protein. The protein of the diet consumed is credited with production of the plasma protein removed except for the *reserve store* of plasma protein related to the previous dietary history and necessarily removed in the initial weeks before a constant output is displayed. These depleted and standardized dogs then react with considerable uniformity to various proteins and are to be considered as biological test machines by which the investigator may evaluate the protein-building worth of various proteins, amino acids, and protein mixtures incorporated in the diet.

Methods

In most respects the procedures used were the same as described in previous papers (8, 15, 13) from this laboratory. The dogs used had received the Laidlaw-Dunkin distemper prophylactic. They were kept in clean metabolism cages with water available at all times. Daily feedings, usually about 5 hours after plasmapheresis, were consumed voluntarily and well, except as noted in the clinical histories. Urine collections were strongly acidified with concentrated sulfuric acid and saved for weekly analysis. Despite precautions fecal contamination did occur and was removed as far as possible by filtration of the sample for analysis.

The various diets are detailed in the clinical histories. In general the basal ration was calculated to contain 0.6 to 1 gm. protein and 70 to 80 calories per kilo of body weight. Most rations included cane sugar or corn syrup (Karo Blue Label), Vitavose (Squibb), lard or cottonseed oil or butter fat (the supernatant and filtered fat from melted creamery butter), and bone ash. All diets contained

canned tomatoes, cod liver oil, and salt mixture (12)—without iron. The figures accepted for protein content of the various articles given in the diet are here given. Except as noted, the determinations were made in this laboratory by macro-Kjeldahl analysis and the protein calculated as 6.25 times the total nitrogen. Protein content: pork kidney, 16.2 per cent; pork liver, 20 per cent; canned salmon (total contents), 19 per cent; beef heart, 16 per cent; Vitavose, 8 per cent and 15 per cent;¹ canned tomato, 1.2 per cent; bran flakes (Post's), 13.2 per cent; boiled potato, 2.5 per cent; beef extract (Liebig), no protein but 9 per cent nitrogen;² serum, concentrated (Lilly), 86 per cent; yeast (Fleischmann), 13 per cent;² dog red blood cells, 33 per cent; soy bean meal, 40 per cent; thyroid powder, U.S.P. (Armour), 41.5 per cent; rice polisibings and beef stomach digest (Lilly), 31.7 per cent. All amino acids used were obtained from Eastman Kodak Company; the specific ones were *l*-cystine, glycine, *d*-glutamic acid, tryptophane, and the dibydrochlorides of *dl*-lysine, *l*-histidine, and *d*-arginine. The rice polisibings-beef stomach digest is the dried and defatted material obtained from acid digestion of equal parts of the fresh materials so concentrated that 1 gm. equals 1 gm. of each of the original substances. 1 gm. of the concentrated serum used contained the material obtained by alcoholic precipitation and drying of about 11 ml. of original serum. Liver extract (Lederle) "for intramuscular use" is said to contain in 1 ml. the active material from 100 gm. of fresh tissue. The soy bean meal used was light brown in color (7) and probably, though not certainly, of the same lot as that previously tested. We do not know how much heat was applied in the process of its preparation. All substances were fed as purchased with the following exceptions: the pork kidney and the beef heart were cooked in a double boiler and fed with the broth; the soy bean meal was cooked with water for 1 hour in a double boiler into a thick mush; pork liver was ground and fed raw; yeast was autoclaved for 15 minutes at 15 pounds steam pressure (256°F.); dog red blood cells were washed in modified Locke's solution, measured when packed by centrifuging, and coagulated with mild heat; potatoes were boiled, peeled, and ground. All the components of the daily diet were thoroughly mixed together before feeding. For production of the sterile abscesses, spirits of turpentine (0.8 ml.) was injected subcutaneously into the lateral thoracic regions, using aseptic precautions.

The procedure of plasmapheresis was carried on as described (8) and modified (13) in previous reports from this laboratory. The chemical methods used also were the same as previously outlined with the following exceptions. Selenium dioxide (in solution, selenous acid) was used as catalyst in place of cupric sulfate in the macro-Kjeldahl digestion mixture (10). Total digestion time was thereby reduced to 75 minutes and distillation was facilitated by lessened foaming. The digestion mixture used contained concentrated sulfuric acid, 20 ml.; potassium sulfate, 10 gm., and selenium, 0.1 gm. (2 cc. of a 7 per cent solution of selenium

¹ As given by manufacturer for different lots.

² Determinations made in Department of Vital Economics.

dioxide). Total nitrogen and albumin nitrogen of the plasma and total urinary nitrogen were all determined with this mixture. Half the quantity of selenium was used in determining plasma non-protein nitrogen, in which procedure trichloroacetic acid was used to precipitate the protein (14). Nitrogen determinations on test food substances and plasma fractions were checked by the established method (using cupric sulfate as catalyst) and the results thus far obtained have indicated the shorter method (employing selenium) to be of equivalent accuracy.

Since all plasma nitrogen determinations were made on samples from the pooled blood from each bleeding, in which samples saturated sodium citrate solution (1 part to 100 parts blood) was the only anticoagulant, *all figures pertaining to circulating plasma nitrogen are low*. When equal portions of the same blood are treated with isotonic sodium oxalate, 1.4 per cent (1 part to 5 parts blood), instead of saturated sodium citrate, it is found that the citrated plasma volume is from 3 per cent to 16 per cent greater than the oxalated, tending to vary directly as the oxalate hematocrits over the range from 40 per cent to 60 per cent. This indicated dilution by hypertonic sodium citrate and the known physiological dilution occurring during large bleedings both operate to produce lower concentrations of elements in the pooled plasma than existed in the animal body. Moreover, the shift of protein from plasma to corpuscles thought to occur upon addition of hyperosmotic solutions to blood *in vitro* (2) would tend further to reduce the plasma protein concentration as given. *The figures for total grams of protein removed remain unaffected.*

EXPERIMENTAL OBSERVATIONS

In the following tables and clinical histories are recorded the complete observations made on two dogs. In all instances, except the "initial sample" at the start of each experiment, the figures are those of weekly totals or averages. A plasmapheresis was performed usually on 6 of the 7 days of each week, although sometimes this procedure on only 5 of the 7 days sufficed to maintain the average blood plasma concentration between 3.80 and 4.30 per cent for the period. During weeks of fasting or of sugar feeding, exchanges were less frequent, and in the experiments with chemical inflammation higher averages were maintained to insure against possible severe depressions of the plasma protein level. As defined in this laboratory, the *potency ratio* of a given substance means the number of units of protein in this substance which are associated with the production of one unit of plasma protein. Obviously the lower the ratio the higher the potency.

In calculating nitrogen balance the weekly loss of nitrogen in feces of 2.7 gm. is based on a determination made during the previous work (13) on these dogs. Large factors in producing the total negative balance for the period of observation are the nitrogen lag or carry over from the higher intake level of kennel diet and the nitrogen in the *reserve store* of plasma protein removed.

In Tables 1 and 1-a are shown 25 weeks of experimentation on a dog (34-152) used successfully in a previously reported test period (13) of 21 weeks. During this former period the basal output averaged close to 12 gm. plasma protein per week and the reserve store, after 26 days on a basal diet followed by 1 week of dextrose feeding, amounted to only 10 gm. plasma protein. The first weeks in the present experiment offer a conspicuous contrast. Careful study of Table 1 indicates that the true basal output per week is 14 gm. plasma protein. It is obvious that this dog has a large *reserve store* of plasma protein-producing materials (56 gm. plasma protein above basal removed in the first 5 weeks). It requires 6 more weeks to remove all of this reserve store. In an attempt to explain this observation we may argue that the dog, on account of the experience with plasmapheresis the year previously, had developed greater capacity to conserve and retain tenaciously this reserve store of protein building material. This hypothesis will be tested in other animals.

The potency ratios for the raw and cooked kidney (Table 1) obviously cannot be determined accurately, as the reserve store has not been completely exhausted and contributes an uncertain amount to the protein output during such periods.

Amino acids if given in proper mixtures should be well utilized to form plasma protein. *Lysine* is well represented in the plasma protein, but given alone with this basal ration it is inert. *Lysine* when given with histidine and arginine (3) does have a slight effect (Table 1, period 19) but only 3.7 gm. plasma protein are produced above the basal control level.

Some observations on milk production in rats (5) suggested a trial of the combination of amino acids represented in glutathione. There does appear to be a definite increase in plasma protein output during and for 3 weeks following the feeding of cystine, glutamic acid, and glycine. Above an estimated basal production of 14 gm. per week

TABLE 1

Blood Plasma Depletion and Regeneration
Kidney Rested. Influence of Certain Amino Acids

Dog 34-152.

Period 7 days	Diet Kidney basal + supplement	Protein intake Total for 7 days	Plasma protein removed Total for 7 days			Protein removed above basal*	Blood plasma Average con- centration	
			Albu- min	Glob- ulin	Total		Total protein	A/G ratio
		gm.	gm.	gm.	gm.	gm.	per cent	
	Kennel						6.50	1.3
1	Dextrose, 420 gm.	0	4.6	3.5	8.1		6.26	1.3
2	Basal	64	20.0	17.9	37.9		5.07	1.1
3	Basal	64	11.2	11.0	22.2	56.3	4.35	1.0
4	Basal	64	13.0	12.8	25.8		4.29	1.0
5	Basal	64	9.0	9.3	18.3		4.13	1.0
6	Basal + kidney (raw) 400+ gm.	129+	15.7	12.9	28.6	24.3	4.12	1.3
7	Basal	64	10.8	9.2	20.0		4.24	1.2
8	Basal	64	9.3	8.4	17.7		4.02	1.1
9	Basal + kidney (cooked) 350 gm.	121	10.7	11.2	21.9	12.4	4.14	1.0
10	Basal	64	7.8	7.9	15.7		4.04	1.0
11	Basal	64	8.4	8.4	16.8		4.07	1.0
12	Basal + lysine, 7 gm.	64	6.5	6.2	12.7		4.02	1.1
13	Basal	64	6.2	6.7	12.9		4.09	0.9
14	Basal + cystine, 7 gm., glu- tamic acid 8.4 gm., and glycine, 42. gm.	64	7.9	7.9	15.8	7.1	4.20	1.0
15	Basal	64	8.4	7.4	15.8		4.17	1.1
16	Basal	64	8.3	7.9	16.2		4.14	1.0
17	Basal	64	7.4	7.9	15.3		4.14	0.9
18	Basal	64	6.5	6.7	13.2		4.19	1.0
19	Basal + histidine, 7 gm., lysine, 7 gm., and argi- nine, 2.8 gm.	64	8.0	8.6	16.6	3.7	4.20	0.9
20	Basal	64	7.0	7.7	14.7		4.09	0.9
21	Basal	64	7.0	7.4	14.4		4.23	0.9
22	Fasting	0	4.5	6.1	10.6		4.23	0.7
23	Basal	64	5.4	8.9	14.3		4.20	0.6
24	Basal + rice polishing-beef stomach digest, 350 gm.	175	7.8	11.7	19.5	14.1	4.29	0.7
25	Basal	63	8.2	14.4	22.6		4.08	0.6

* Estimated basal output equivalent to 14 gm. plasma protein per week.

TABLE 1-a
Weight, Nitrogen Balance, and Blood Findings

Dog 34-152.

Period 7 days	Diet Kidney basal + supplement	Weight	N intake	N in plasma removed	Urinary N	Nega- tive N balance	R.B.C. hemato- crit	Plasma volume
		kg.	gm.	gm.	gm.	gm.	per cent	cc.
	Kennel	15.1					51.5	652
1	Dextrose, 420 gm.	14.5	0.0	1.3	9.7	13.7	49.8	631
2	Basal	13.9	10.2	6.1	12.4	11.0	45.3	—
3	Basal	14.0	10.2	3.6	10.9	7.0	43.8	588
4	Basal	14.0	10.2	4.1	10.5	7.1	49.5	636
5	Basal	14.0	10.2	2.9	11.0	6.4	49.9	614
6	Basal + kidney, 400+ gm.	14.1	20.6+	4.6	10.1	+3.2	51.2	704
7	Basal	14.2	10.2	3.2	8.1	3.8	51.2	664
8	Basal	14.0	10.2	2.8	9.2	4.5	51.3	572
9	Basal + kidney, 350 gm.	14.1	19.3	3.5	10.6	+2.5	50.4	622
10	Basal	14.1	10.2	2.5	9.5	4.5	48.3	—
11	Basal	14.3	10.2	2.7	6.7	1.9	48.5	590
12	Basal + lysine, 7 gm.	14.1	11.5	2.0	8.8	2.0	49.1	555
13	Basal	14.2	10.2	2.1	7.2	1.8	51.4	582
14	Basal + cystine, 7 gm., glu- tamic acid, 8.4 gm., gly- cine, 4.2 gm.	14.5	12.6	2.5	6.2	+1.2	51.8	553
15	Basal	14.5	10.2	2.5	7.7	2.7	51.7	589
16	Basal	14.8	10.2	2.6	5.8	0.9	51.3	625
17	Basal	14.8	10.2	2.5	8.0	3.0	51.9	555
18	Basal	14.9	10.2	2.1	8.0	2.6	52.5	571
19	Basal + histidine, 7 gm., lysine, 7 gm., arginine, 2.8 gm.	14.9	14.3	2.7	11.2	2.3	52.0	589
20	Basal	15.0	10.2	2.4	8.1	3.0	52.4	—
21	Basal	15.1	10.2	2.3	9.4	4.2	54.3	620
22	Fasting	14.3	0.0	1.7	9.8	14.2	54.8	460
23	Basal	14.4	10.2	2.3	8.1	2.9	53.4	573
24	Basal + rice polishings-stom- ach digest, 350 gm.	14.5	28.0	3.1	13.1	+9.1	52.1	587
25	Basal	14.7	10.1	3.6	10.9	7.1	50.6	—

the *plasma protein output* referable to this amino acid supplement (period 14) is 7.1 gm. What fluctuations occurred in plasma volume and protein concentration favored a slight increase in the mass of circulating plasma protein. The animal gained more than 0.5 kg. in weight during this 4 weeks' period and displayed a decrease in

urinary nitrogen. There was a positive nitrogen balance about equivalent to that noted after feeding 350 gm. cooked kidney. This all speaks for utilization of these amino acids in protein metabolism. In contrast, during the week of histidine, lysine, and arginine feeding (period 19) the urinary nitrogen *increased* almost to the amount of the excess nitrogen intake.

The complication of spoon feeding was introduced in the 20th period because the dog refused its food. A period of fasting did not improve the appetite, although at all times the animal readily ate the diet when spoon fed. The fasting output (period 22) was apparently higher than usual, but on deducting 5.7 gm. accounted for by the conspicuous shrinkage of plasma volume, the usual output is noted.

A digest of beef stomach and rice polishings (period 24, Table 1) displays an expected capacity for plasma protein production. Despite the fact that the experiment was terminated after 5 days of the second after period, a fairly high potency ratio (7.9) was already indicated. Too much weight cannot be given to this test of the rice polishings digest, as the subsequent acute fatal infection may have been developing during this period.

We note a sharp drop in the A/G ratio during the last four periods (Table 1). This drop frequently indicates trouble. Apparently the capacity to form albumin is more sensitive to harmful factors than is the capacity to produce globulin. During the last week of life a rapid sedimentation rate of the red cells was noted as a further index of trouble.

The total *negative nitrogen balance* for the 25 weeks amounts to 90.6 gm. There is little weight loss. Obviously there has been a serious depletion of the essential protein matrix of the body cells, and this may well be an important influence in lowering resistance to bacterial infection. The red cell hematocrit showed no significant fall at any time, excluding anemia as a possible cause of lowered resistance in spite of all the bleeding.

Clinical History.—Dog 34-152 (Tables 1 and 1-a). An adult male mongrel hound weighing 15.1 kg. had been previously tested over a period of 21 weeks (13) and had subsequently rested on kennel diet for 16 weeks. The dog was fasted during the 1st week except for the daily administration of 60 gm. dextrose in 200 ml. water by stomach tube. The basal daily diet then provided was the same

as that used during the previous test period (13), except for a deduction of 10 gm. sugar and the addition of 7 gm. lard. It contained 50 gm. (raw weight) cooked pork kidney (8.1 gm. protein); 25 gm. canned tomato (0.3 gm. protein); 5 gm. Vitavose (0.4 to 0.75 gm. protein); 120 gm. cane sugar; 15 gm. cod liver oil; 37 gm. lard; 10 gm. hutter fat; 5 gm. bone ash; 1 gm. salt mixture. This diet furnished about 75 calories per kilo body weight daily and had a bulk of 200 ml. The daily diet was voluntarily consumed 100 per cent until the middle of the 19th week. From then on much or all of the diet had to be spoon fed. This procedure met with no resistance and the food was swallowed 100 per cent. Except for some decrease in spontaneous activity, no change in the animal's clinical condition was detected at this time. With hope of stimulating the appetite and in view of a gradual weight gain, 10 gm. cod liver oil was deducted from the daily diet during the last 6 weeks. On one occasion only, the day before the end of the last recorded week, an estimated 15 per cent of the day's diet was regurgitated. 2 days later a rapid *sedimentation rate* of the blood drawn for sampling was noted. The plasma protein level on this day was 3.87 per cent. Outspoken signs of illness were absent at this time, although the dog did appear below par. Then followed a progressively rapid downhill course through irritability, stupor, and convulsions to death on the 5th day after the end of the 25th week.

Autopsy disclosed acute vegetative endocarditis; focal acute myocarditis with abscess formation; acute suppurative nephritis; infected thrombus partially occluding left common iliac artery; bronchopneumonia; splenic infarct; cerebral hemorrhage; hemosiderin deposits in lymph nodes, spleen, liver, and bone marrow.

During the course of the experiment 8665 ml. of red blood cells were withdrawn and 10,092 ml. were returned suspended in modified Locke's solution containing a total of 541 gm. glucose. Weekly, non-protein nitrogen determinations varied from 15 to 25 mg. per cent during the entire period. The first supplement of kidney in period 6 was an accident and the amount of raw kidney was a little in excess of 400 gm. The second supplement of kidney was cooked and mixed with the basal ration over the 7 day period. The final supplement in Tables 1 and 1-a was a digest of beef stomach and rice polisblings described under Methods.

Tables 2 and 2-a present the results of satisfactory tests over a continuous period of 26 weeks. When these observations were begun the dog (33-11) had been on kennel diet for 16 weeks following a previously reported experiment (13) of only 6 weeks' duration. The initial level of blood plasma protein concentration (6.09 gm. per cent) was 0.75 gm. per cent higher than that of the previous experiment, and the reserve store of plasma protein building material was just 4 times greater (34.1 gm.). It appears that both animals acquired larger protein reserves and higher plasma protein concentration levels following plasmapheresis experiments done 4 months previously. On

TABLE 2

*Blood Plasma Depletion and Regeneration
Dried Serum, Yeast, Thyroid, Hemoglobin Decreasingly Potent
Iron or Intramuscular Liver Extract Inert*

Dog 33-11.

Period 7 days	Diet Salmon and kidney basals + supplements	Protein intake Total for 7 days	Plasma protein removed Total for 7 days			Protein re- moved above basal*	Potency ratio Protein intake to protein output	Blood plasma Average concentration	
			Albu- min	Glob- ulin	Total			Total pro- tein	A/G ratio
		gm.	gm.	gm.	gm.	gm.		per cent	
	Kennel							6.09	—
1	Dextrose, 350 gm.	0	4.4	3.3	7.7			5.80	1.5
2	Kidney basal	64	14.0	8.4	22.4			5.03	1.7
3	Kidney basal	64	12.5	9.0	21.5			4.20	1.4
4	Kidney basal	64	9.9	8.6	18.5	34.1		4.44	1.1
5	Salmon basal A	47	6.7	7.1	13.8			4.01	0.9
6	Salmon basal A	47	4.8	5.1	9.9			3.98	0.9
7	Salmon basal B	74	6.7	5.3	12.0			4.25	1.2
8	Salmon basal B + yeast, 450 gm.	133	9.9	8.1	18.0	13.4	4.4	4.21	1.2
9	Salmon basal B	74	10.2	7.7	17.9			4.09	1.3
10	Salmon basal B	74	7.7	5.8	13.5			3.93	1.3
11	Dextrose, 350 gm. + iron, 1.4 gm.	0	5.0	4.8	9.8			4.13	1.1
12	Salmon basal B	74	5.1	5.3	10.4			4.00	1.0
13	Salmon B + liver extract (parenteral)	74	6.1	6.4	12.5			4.01	1.0
14	Salmon basal B	74	6.3	5.9	12.2			4.21	1.1
15	Salmon B + red blood cells, 218 gm.	146	9.3	8.1	17.4	7.1	10.1	4.14	1.2
16	Salmon basal B	74	7.5	6.2	13.7			4.06	1.3
17	Salmon basal B + iron, 1.4 gm.	74	6.8	5.6	12.4			4.13	1.2
18	Salmon basal B	74	5.5	5.4	10.9			4.07	1.0
19	Salmon B + dried serum, 35 gm.	104	8.8	7.9	16.7	8.5	3.5—	4.10	1.1
20	Salmon basal B	63±	8.1	7.7	15.8			4.16	1.1
21	Kidney basal	64	2.8	2.8	5.6			3.80	1.0
22	Kidney basal	64	5.3	6.0	11.3			3.98	1.0
23	Salmon B + soy bean, 175 gm.	144	8.8	9.4	18.2	5.8	12.1	4.13	0.9
24	Salmon basal B	74	5.7	5.9	11.6			3.95	1.0
25	Salmon basal B + thyroid, 70 gm.	103	7.3	8.1	15.4	5.5	5.3	4.32	0.9
26	Salmon basal B	74	6.7	7.4	14.1			4.01	0.9

* Estimated basal output equivalent to 12 gm. plasma protein per week.

TABLE 2-a
Weight, Nitrogen Balance, and Blood Findings

Dog 33-11.

Period 7 days	Diet	Weight	N intake	N in plasma re- moved	Uri- nary N	Nega- tive N balance	R.B.C. hema- tocrit	Plasma volume
		kg.	gm.	gm.	gm.	gm.	per cent	cc.
	Kennel	12.5					56.8	383
1	Dextrose, 350 gm.	11.9	0.0	1.2	18.7	22.6	53.4	396
2	Kidney basal	11.3	10.2	3.5	15.2	11.2	46.3	396
3	Kidney basal	11.4	10.2	3.4	10.6	6.5	44.5	411
4	Kidney basal	11.5	10.2	3.0	11.0	6.5	48.9	426
5	Salmon basal A	11.6	7.5	2.2	10.5	7.9	49.6	444
6	Salmon basal A	11.6	7.5	1.6	9.4	6.2	46.2	367
7	Salmon basal B	11.5	11.8	1.9	10.2	3.0	49.6	387
8	Salmon basal B + yeast, 450 gm.	11.6	21.3	2.9	13.4	+2.3	50.0	419
9	Salmon basal B	11.6	11.8	2.9	11.1	4.9	49.1	402
10	Salmon basal B	11.6	11.8	2.2	8.9	2.0	49.0	395
11	Dextrose, 350 gm. + iron, 1.4 gm.	11.1	0.0	1.6	7.0	11.3	48.8	353
12	Salmon basal B	11.1	11.8	1.7	7.9	0.5	47.2	391
13	Salmon B + liver extract (parenteral)	11.2	11.8	2.0	9.8	2.7	49.6	344
14	Salmon basal B	11.3	11.8	1.9	9.4	2.2	49.7	409
15	Salmon B + red blood cells, 200 cc.	11.4	23.4	2.8	13.6	+4.3	49.4	—
16	Salmon basal B	11.4	11.8	2.2	9.1	2.2	52.2	414
17	Salmon basal B + iron, 1.4 gm.	11.5	11.8	2.0	9.3	2.2	52.4	376
18	Salmon basal B	11.5	11.8	1.7	8.7	1.3	51.8	360
19	Salmon B + dried serum, 35 gm.	11.7	16.6	2.7	9.7	+1.5	51.5	389
20	Salmon basal B	11.6	10.1	2.5	6.6	1.7	52.7	372
21	Kidney basal	11.8	10.2	0.9	7.0	0.4	51.1	445
22	Kidney basal	11.7	10.2	1.8	7.9	2.2	51.9	358
23	Salmon B + soy bean, 175 gm.	11.9	23.0	2.9	13.0	+4.4	53.1	396
24	Salmon basal B	12.2	11.8	1.9	9.7	2.5	52.8	409
25	Salmon basal B + thyroid, 70 gm.	11.9	17.1*	2.5	12.5	0.6	52.6	345
26	Salmon basal B	11.8	11.8	2.3	9.6	2.8	54.0	390

* Includes nitrogen in 6 gm. of beef extract.

the salmon basal diet the estimated basal protein output equaled that on the kidney basal (about 12 gm.) but the salmon is less efficient with its 16 per cent larger protein content.

The autoclaved yeast was readily consumed by the dog and produced none of the gastro-intestinal disturbance noted with the non-autoclaved material (13). Each 4.4 gm. of yeast protein resulted in the production of 1 gm. of plasma protein. The carry over into the week after feeding was equal to the output of the feeding week.

Two attempts to get further information on a possible relationship of oral iron to plasma protein regeneration tend to deny a direct relationship. A previous test (15) adding 2 gm. ferric citrate (360 mg. iron) daily to a vegetable protein diet was accompanied by an excess protein output of 14.4 gm. but this was associated with anemia. This quantity is too large to be accounted for by any experimental variation such as blood volume fluctuation. In the present experiments during one period 1.15 gm. ferric citrate scales (200 mg. iron) in solution were given with 50 gm. dextrose daily by stomach tube (period 11, Table 2). The output of 9.8 gm. exceeds the expected fasting output but little, and the following week on basal diet yields less than the basal output. When the same amount of ferric citrate was added to the basal diet during period 17, the result was negative.

A clinical observation reported to us (by Dr. D. J. Stephens) occasioned the trial of intramuscular liver extract. No effect on plasma protein regeneration was indicated. Oral liver extract enabled protein production in proportion to its protein content (15); the product for intramuscular use contained no protein.

Proteins in *red blood cells* (about 95 per cent hemoglobin) have a low potency ratio of 10. Hemoglobin when fed to anemic dogs has a potency ratio of 10, that is, one must feed about 100 gm. hemoglobin to produce 10 gm. new hemoglobin in red cells (19).

Dried serum, 1 gm. of which is obtained by alcoholic precipitation from about 11 ml. original serum, demonstrated excellent capacity for plasma protein production (potency ratio 3.5) despite some gastric disturbance, which in the following week resulted in the loss by vomiting of an amount estimated to equal 1 day's diet. A depression in plasma regeneration to a fasting level (period 21, Table 2) followed this upset and may have been due in part to the gastro-intestinal

disturbance. The change from salmon to kidney basal may have had an influence in the depression, or a slowly developing deficiency state may have become manifest.

Soy bean meal (period 23, Table 2) retested at this time showed the expected prompt effect without carry over, but the total plasma protein output was less than in a previous experiment (13). We have observed before that the potency ratio of a given food factor may remain unchanged or may show considerable change when tested with different basal diets. The soy bean meal with a kidney basal showed a potency ratio of 7.1 in contrast to this experiment with a salmon basal and potency ratio of 12.1. The soy bean feeding did favor a weight increase. A possible deficiency state may also have been a factor in this reaction.

Thyroid powder given in large doses (period 25, Table 2) will accelerate metabolism, and it was thought that such acceleration might increase or decrease plasma protein production, depending upon the site of action. As a matter of fact, there is no change in the picture and the protein of the thyroid powder is utilized just like any other protein, with a potency ratio of 5.3, indicating excellent utilization. It will be useful to know the reaction to thyroxin alone.

During periods 27 to 31, Table 3, Dog 33-11 was progressing into a serious deficiency state due to inadequate protein intake and a steady drain on the plasma protein. The basal diet no longer enabled the dog to produce 12 gm. plasma protein each week. The dog had a distaste for food necessitating spoon feeding with occasional regurgitation, and there developed superficial ulcers on the skin over the gluteal regions. There was considerable loss of hair but no weight loss. There was a definite fall in the A/G ratio.

The amino acid feeding (period 28, Table 3), gave no significant change in the plasma protein output, but this may be a false picture because of the deficiency state. The positive nitrogen balance suggests some utilization of some of these amino acids (compare period 14, Table 1-a).

Periods 37 and 39, Tables 3 and 3-a, present experiments on the effects of inflammation upon plasma protein regeneration. Several previous observations (13) have been made on the influence of unwanted bacterial infection arising in the course of an experiment.

This influence has been uniformly depressant to plasma protein formation. In the present observations local inflammation with abscess production and a certain degree of accompanying general intoxication has been produced by the chemical irritation of turpentine injected

TABLE 3
Blood Plasma Depletion and Regeneration
Influence of Sterile Abscess

Dog 33-11.

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein removed Total for 7 days			Protein output Per cent of protein intake	Protein output Per cent of esti- mated basal output*	Blood plasma Average concentration	
			Albu- min	Glob- ulin	Total			Total pro- tein	A/G ratio
		gm.	gm.	gm.	gm.			percent	
27	Salmon basal B	74	4.2	5.0	9.2			3.81	0.8
28	Salmon B + tryptophane, 7 gm., cystine, 7 gm., glutamic acid, 8.4 gm., glycine, 2.8 gm.	72	3.0	3.9	6.9			3.89	0.7
29	Salmon basal B	58±	2.8	3.6	6.4			3.79	0.8
30	Potato-bran	68	1.4	1.7	3.1			3.85	0.8
31	Potato-heart	41	2.3	3.2	5.5			4.16	0.7
	Raw liver A	86							
32	Raw liver A	150	2.3	3.0	5.3			4.39	0.8
33	Raw liver A	150	5.5	6.3	11.8			4.69	0.9
34	Raw liver A	150	11.7	12.8	24.5			4.70	0.9
35	Raw liver B	115	10.9	13.5	24.4	21.2	101.0	4.46	0.8
36	Raw liver B	115	10.4	13.9	24.3	21.1	100.5	4.27	0.7
37†	Basal + turpentine abscess	78	1.8	3.0	4.8	6.2	29.5	4.06	0.6
38‡	Raw liver B	129	9.2	14.8	24.0	18.6	88.6	4.33	0.6
	Raw liver C								
39	C + turpentine abscesses	108	3.0	5.3	8.3	7.7	36.7	4.46	0.6
40	Raw liver C	107	9.1	14.8	23.9	22.3	106.2	4.54	0.6

* Estimated basal output on raw liver B diet equivalent to 21 per cent of intake.

† Period of 5 days.

‡ Period of 8 days.

subcutaneously. Systemic reaction is limited usually to the first 3 days after the injection of the turpentine and is marked by a mild reduction in activity and appetite and a sharp leucocytosis and fever. The urinary nitrogen is increased. With the walling off of the in-

flammatory area the general reaction subsides and uncomplicated healing begins immediately with the evacuation of the abscess on the 4th or 5th day. This limited, acute, controlled, inflammatory process affords a very satisfactory test period. Observations on two such periods (37 and 39) are listed in the tables (3 and 3-a).

TABLE 3-a

Weight, Nitrogen Balance, and Blood Findings

Dog 33-11.

Period 7 days	Diet	Weight	N intake	N in plasma re- moved	Uri- nary N	Negative N balance	R.B.C. hema- to- crit	Plasma volume
		kg.	gm.	gm.	gm.	gm.	per cent	ml.
27	Salmon basal B	11.9	11.8	1.5	9.0	1.4	54.7	376
28	Salmon B + tryptophane, 7 gm., cystine, 7 gm., glutamic acid, 8.4 gm., glycine, 2.8 gm.	11.9	14.9	1.1	5.7	+5.4	52.7	381
29	Salmon basal B	11.6	9.3±	1.0	7.2	1.6±	51.5	380
30	Potato-bran	11.2	10.9	0.5	7.9	0.2	48.2	424
31	Potato-heart	11.4	20.4	0.9	8.7	+8.1	46.2	388
	Raw liver A							
32	Raw liver A	11.6	25.0	0.9	10.5	+10.9	50.9	510
33	Raw liver A	11.7	25.0	1.9	12.9	+7.5	54.9	—
34	Raw liver A	11.8	25.0	3.9	14.2	+4.2	52.9	—
35	Raw liver B	12.0	18.4	3.9	13.1	1.3	52.8	—
36	Raw liver B	12.1	18.4	3.9	8.8	+3.0	52.2	—
37*	B + turpentine abscess	12.4	12.5	0.8			48.7	—
38†	Raw liver B	12.3	20.6	3.8	19.7‡	+3.1§	49.6	—
	Raw liver C							
39	C + turpentine abscesses	12.4	17.3	1.3	13.7	1.4§	53.5	—
40	Raw liver C	12.2	17.1	3.8	12.0	1.4	52.5	—

* Period of 5 days.

† Period of 8 days.

‡ Period 37 plus period 38.

§ Include abscess nitrogen, a total of approximately 1.7 gm. for the 2 periods (4).

The *raw liver diet* was chosen to favor a fairly large basal output of plasma protein and to be highly tempting to the dog during the periods of the experimental inflammation. The 37th period shows a marked depression of plasma protein regeneration and a conspicuous decline in the average blood plasma protein concentration accompanying

the production of one turpentine abscess. The figures indicate a drop in protein output to 29 per cent of the output of the previous (36th) week but a part of this drop is due to a decreased food intake. In the second abscess period (39th period) the process was prolonged to a full week by the production of two consecutive abscesses (Clinical

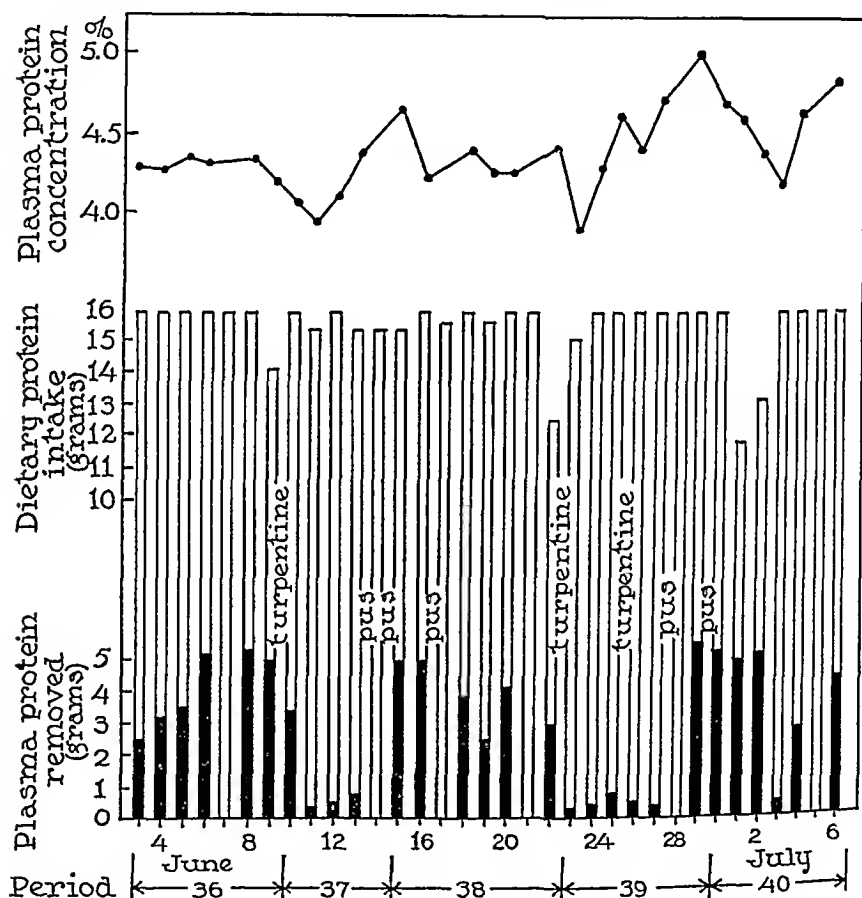


CHART A. Plasma protein production depressed by turpentine abscess.

History, Dog 33-11). Depression in plasma protein regeneration was marked (36.5 per cent of that for the 36th week), but not as great as that during the first abscess and the average circulating plasma protein level was higher. This elevation in output and the further relative elevation of the 40th week may indicate accumulation of undepleted

protein and protein building materials during the 38th period, or may indicate a delayed output based on protein building materials accumulated during the abscess periods themselves. The urinary nitrogen was distinctly elevated during the second abscess period and remained high during the final week on account of the hemoglobinuria (Clinical History, Dog 33-11).

Clinical History.—Dog 33-11 (Tables 2 and 2-a, 3 and 3-a; Chart A). An adult female mongrel, born Nov. 25, 1932, in this laboratory, was raised on a salmon-bread-apricot diet. 2 months in the anemia colony were followed by 12 months on kennel diet. Its initiation to plasmapheresis continued only 5 weeks and has been reported (13). Some 16 weeks on kennel diet followed this depletion. Oct. 1, 1935, found the various initial determinations as given (Tables 2 and 2-a). During the 1st week 50 gm. dextrose in 150 ml. water were given daily by stomach tube. The kidney basal ration provided this dog was the same as that given Dog 34-152 (Table 1), except for reduction of cane sugar to 95 gm., lard to 30 gm., and cod liver oil to 10 gm. The salmon basal diets A and B were identical with the kidney basal ration, except for the substitution for the kidney of canned salmon, 20 gm. in A and 50 gm. in B. The latter daily diet contained 10.5 gm. protein and 926 calories. Weight was maintained and the diets were readily eaten. The yeast was weighed before autoclaving. In the 11th week 25 ml. of 4.6 per cent solution of ferric citrate scales (Merck) (17.4 per cent iron) were added to the dextrose given daily as in the 1st week, and in the 17th period the same quantity of iron was added to the basal diet. The liver extract was given intramuscularly, 1 ml. each day. The gastro-intestinal disturbances from the 19th to the 29th weeks, apparently related to the feeding, have been mentioned above. Beef extract tried in the 25th period was unsuccessful in overcoming dislike for the diet. Spoon feeding was largely successful, although, as indicated in the figures for protein intake, food was sometimes lost by regurgitation. The non-protein nitrogen varied from 12 to 24 mg. per cent in the first 25 weeks and from 23 to 32 mg. per cent in the following 15 weeks.

Upon changing in the 30th week to the potato-bran diet the dog voluntarily returned to 100 per cent food consumption for four days and then began to lose appetite. This diet contained 200 gm. boiled potato (5 gm. protein); 35 gm. bran flakes (4.6 gm. protein); 25 gm. tomato (0.3 gm. protein); 61 gm. corn syrup; 20 gm. cod liver oil; 20 gm. cottonseed oil; 1 gm. salt mixture. For 3 days in the 31st period 50 gm. beef heart (8.0 gm. protein) and 5 gm. Vitavose (0.75 gm. protein) were substituted in the above diet for the bran flakes, but still the response was poor.

From the 1st day the dog ate the *raw liver diet* avidly. Within 3 weeks the sores on the buttocks were completely healed and loss of hair appeared much reduced. The first liver diet consisted of 100 gm. raw liver (20 gm. protein); 10 gm. Vitavose; 50 gm. tomato; 94 gm. cane sugar; 20 gm. cod liver oil; 20 gm. cotton-

seed oil; 15 gm. bone ash; 1 gm. salt mixture. The B modification reduced the liver to 75 gm. and increased the sugar to 102 gm., maintaining a total caloric value of 903. The C modification maintained the same protein kind and quantity but reduced the caloric value to 800, and arrested the gradual weight increase. The animal maintained a good clinical condition despite the abscess production, although during these periods it frequently left a small amount of its diet and rarely regurgitated a small quantity.

To produce a sterile abscess turpentine was injected on the right side on the last day of the 36th period. Leucocyte count was 12,000 before injection and 56,000 2 days after. Rectal temperature was 41.3°C. $\frac{1}{2}$ hour following plasmapheresis on the 1st day of the 37th period, but fell to 38.9°C. within 4 hours. The remaining 4 days of the period were without fever or plasmapheresis. On the 3rd day the local swelling was fluctuant and on the 4th day the abscess was drained by incision. 80 ml. of sanguineous seropurulent material were obtained. The leucocyte count dropped to 14,800 on this day. Culture of the abscess material produced no bacterial growth. The following day similar but thinner fluid, about 35 ml., was removed. Turpentine was evident in all this material, as well as in 17 ml. similar material aspirated 2 days later after the wound had closed. On this day, the 2nd of the 38th period, the leucocyte count rose to 39,400 and the temperature climbed to 40.2°C. shortly after plasmapheresis but dropped to 38° less than 4 hours later. During the balance of the period the leucocyte count averaged 25,000 and the rectal temperature did not exceed 38.2°C.

A second abscess was produced with turpentine on the left side on the last day of this period (period 38). Fluctuation in the abscess appeared on the 3rd day of the period and another turpentine injection was made on the right side. On the 5th day the second abscess was drained, yielding 77 ml. seropurulent material and on the 7th day 82 ml. of material was obtained from the third abscess. The leucocyte count reached 53,400 on the 5th day and the temperature 39.8°C. Both of these abscesses healed without further accumulation of fluid, and the leucocyte count gradually dropped to 11,200 by the last day of the 40th period. During the last period transitory pyrexia as high as 40.1°C. would sometimes appear after plasmapheresis, without any recognizable general disturbance.

On the 2nd day of the last period the red cells (131 ml.) prepared for injection were accidentally much overheated, then subsequently cooled to body temperature and injected. Hemoglobinuria was noted within 1 hour and few hours later slight icterus of sclerae and mucous membranes could be detected. No general disturbance in the behavior of the dog was recognized at any time, although part of the diet was refused for 2 days.

The dog was continued on the same diet for 14 days following the discontinuance of plasmapheresis at the close of the 40th period. On the 1st day the blood plasma protein concentration was 4.45 gm. per 100 ml. plasma; the 4th day it had risen to 4.68, the 8th day to 4.80, and the 14th day to 5.87.

DISCUSSION

Infection and its influence on plasma protein production invite discussion. The "sterile abscess" due to turpentine gives the complete clinical picture of a bacterial abscess—*inflammation*, fever, leucocytosis, localized pus formation, and an increased urinary nitrogen. Moreover, it can be promptly terminated on the 3rd or 4th day, with subsequent rapid healing. Chart A shows that the sterile abscess causes a diminution in the production of new plasma protein during the abscess week. A small part of this initial reaction can be explained by extravasation at the site of the abscess and in the first abscess period the slight decrease in protein intake will explain a small part. The larger part is yet to be explained. We might argue that materials which would go to form the plasma proteins were deviated to repair the body tissues whose injury released the customarily noted (4) excess urinary nitrogen. One might also argue that the protein-forming mechanism (in the liver?) is disturbed by the abscess intoxication slowing up plasma protein formation. In the anemic dog this last thesis has been shown to obtain for the inhibition of hemoglobin formation caused by a sterile abscess (17).

Reserve stores of materials which can promptly be converted into plasma proteins have been demonstrated in dogs. The amount of such stores depends in part on the diets of the preceding weeks. It would seem (Tables 1 and 2) that dogs which have been through periods of depletion by plasmapheresis, tend to heap up *greater reserve stores* of plasma protein building materials during intervening rest periods. Where these reserve stores are located is of some interest, and there seems to be no reasonable doubt that a part at least is stored in the liver but the liver cannot possibly hold all such large reserve stores as demonstrated in some dogs. The reserve store may amount to more protein than is contained in the entire liver and we may suspect the muscles as another possible depot (1). Addis and associates (1) and Luck (11) have given evidence for liver storage of proteins related to diet.

Albumin-globulin ratios are much talked about but they scarcely deserve this attention. There is always the uncertainty about the

separation of albumin and globulin into distinct fractions. Some methods used will give gross inaccuracies. A favorite argument is that a drop in the A/G ratio indicates a decreased albumin production and that the particular tissue concerned in the injury reaction is therefore responsible for the manufacture of the albumin of the plasma. For example Dalla Volta (18) notes a fall in the A/G ratio after x-ray injury of the bone marrow and like many others argues that the albumin is formed in the red marrow. *Any considerable disturbance* of the normal state in the dog will show a drop in the A/G ratio and a diet rich in some grain proteins will likewise cause a drop in the A/G ratio (13). Of the plasma proteins the *fibrinogen* is most labile and can be raised or lowered by a great variety of body changes (6). Albumin appears to be more labile than globulin and certainly escapes from the circulation more readily than does the larger globulin molecule—a good example is nephrosis with escape of albumin in the urine. Certain proteins in the food favor albumin production—for example muscle, liver, kidney, soy bean meal. In the present unsatisfactory state of our knowledge too much weight cannot be placed safely upon the interesting fluctuations of the albumin and globulin in the plasma.

The *potency ratio* as used in our papers means the grams of protein fed which will yield 1 gm. of new plasma protein in these depleted dogs. Potency ratios may vary depending upon a number of factors. The basal diet is probably most important as all our basal rations contain some protein which might be capable of supplementing an added protein (*e.g.* liver) but the protein of another basal ration might not act favorably. Presumably the new plasma protein results from the assembly of many amino acids and other materials (in the liver?) and the amount and character of the amino acids coming from one basal diet might determine the reaction with the amino acids coming from some accessory food protein. It is probable also that these dogs can utilize relatively small amounts of added protein more efficiently than larger supplements. The potency ratio would then rise as larger amounts of the food protein were added. The caloric intake may also be a factor. On a given diet it is probable that some dogs can make plasma protein more efficiently than others just as some dogs can make hemoglobin in anemia more efficiently on a given diet as compared with certain other dogs. Similar variations apply to

endurance, speed of running, resistance to infection, and other individual qualities. Obviously the potency ratio must be interpreted cautiously with understanding of all the conditions of the experiment, realizing that apparently insignificant variables may cause sizable differences in reaction. To show the potency ratio of *liver* we refer to Table 4 illustrating relatively stable potency ratios in different dogs under a variety of conditions.

Raw liver was given in Table 3 and this potency ratio of 5.5 may mean a more complete utilization of the uncooked tissue. On the other hand this dog was emerging from a deficiency state and may have been utilizing this protein at an even greater pace than during other more normal periods.

TABLE 4
Potency of Liver in Various Dietary Régimes

Diet	Reference	Dog	Weight of dog	Daily dietary intake			Potency ratio
				Basal + liver		Liver protein total	
				Calories per kg.	Total protein per kg.		
			kg.		gm.	gm.	
Liver added to potato-bran basal...	(15)	32-130	13.4	99	5.8	60.0	6.5
Liver added to kidney basal.....	(13)	34-152	13.2	90	1.7	14.3	6.4
Liver sole article of diet.....	(8)	32-30	8.6	45	7.0	60.0	6.6
Liver 91 per cent of protein in diet..	Table 3	33-11	12.0	75	1.4	15.0	5.5

Gastro-intestinal disturbances are obviously important in this type of experiment and this was to be expected. Fresh yeast causes diarrhea and intestinal disturbance without obvious clinical intoxication but with a great fall in the plasma protein output. There is a conspicuous difference in the utilization of autoclaved yeast, which is well digested and gives a potency ratio of about 4.4. The fresh yeast caused a fall of plasma protein production to the fasting level (Table 5, Reference 13). Likewise in Dog 33-11 (Table 2, period 21) there was some gastro-intestinal disturbance which was probably in part responsible for this fall in the plasma protein output to a fasting level.

Amino acids in plasma depletion experiments cannot fail to intrigue the investigator. Theoretically it should be possible with the proper

mixture of amino acids to influence profoundly the plasma protein production. Such good fortune as yet has not been attained by our few experiments. A combination of cystine, glutamic acid, and glycine by mouth does have a slight influence on new plasma protein production and shows a positive nitrogen balance in this dog (Table 1). Much more work in this field is badly needed.

Iron when given with a potato-bran basal diet (15) in the presence of a moderate anemia did appear to have a definite influence upon plasma protein regeneration. When iron is given with a salmon basal diet or during fasting with a normal hemoglobin concentration (Table 2) there is no effect upon the plasma protein production. We cannot give any adequate explanation for this difference in the action of iron but hope to report in more detail later.

SUMMARY

When blood plasma proteins are depleted by bleeding, with return of washed red cells (plasmapheresis), it is possible to bring dogs to a steady state of low plasma protein in the circulation and a uniform plasma protein production on a basal diet. Such dogs become test subjects by which the effect of various factors on plasma protein regeneration can be measured.

Dogs previously the subjects of plasmapheresis, during long rest periods appear to increase their stores of plasma protein building materials and their blood plasma protein concentrations above former normal levels.

A sterile abscess (turpentine) induces a marked reduction in plasma protein regeneration in these test dogs consuming an ample basal diet. The sharp reduction during the initial 24 hours may in part reflect an extravasation of plasma protein into the injured tissue but there also appears to develop a true disturbance of the mechanism which produces plasma proteins.

Digestive disturbances interfere seriously with plasma protein production. Whereas large quantities of live yeast upset digestion and form no plasma protein, autoclaved yeast is well utilized, having a potency ratio of 4.4.

Amino acids have been tested inadequately. A mixture of cystine,

LIVER FUNCTION AND BLOOD PLASMA PROTEIN FORMATION

NORMAL AND ECK FISTULA DOGS

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The experimental data given below support the thesis that the liver is actively concerned with the fabrication of the plasma proteins. One of the plasma proteins (fibrinogen) takes its origin from the liver and the evidence (5, 3, 8) for this statement is now generally accepted. There is a reserve store of protein material in the liver (1, 7), and some of this or other material is thrown into the circulation after a rapid and extreme plasma depletion (6) to effect a sharp rise in total plasma protein concentration. Liver injury and the Eck fistula abolish this sharp reaction. Disease of the liver in man may show a low plasma protein concentration (12).

The *Eck fistula* permits the portal blood to flow directly into the vena cava and excludes it from the liver so that the blood supply of the liver is then wholly arterial and probably reduced to 25 to 35 per cent of the normal combined portal and arterial flow. Under such circumstances we may have a clinically normal dog, active and lively, with a good appetite, constant weight, and normal gastro-intestinal activity. These dogs often have more or less conspicuous transient clinical disturbances, which become familiar to laboratory workers. Abnormal thirst and diuresis may appear (2). At first dogs may be intoxicated with meat feeding but this abnormality usually disappears. There may be a slight icteric tinge in the blood plasma. The liver is distinctly smaller than normal and we have evidence that liver function may be subnormal (15). It has been shown that the blood ammonia in such dogs on a high protein diet may be increased. Further-

more blood ammonia values after ammonia administration are higher in Eck fistula dogs than in normal controls (11). This indicates that the ability of the liver to form urea is subnormal in the Eck fistula dog. Abnormalities related to the function of the liver in carbohydrate metabolism have been recognized.

Perhaps it is surprising that the Eck fistula liver with its limited blood supply can carry on so effectively its metabolic duties. The well known margin of safety of the liver is probably responsible. It seemed that an extra burden placed on such an Eck fistula liver might well bring out its incompetency and we planned to test its plasma protein-forming capacity by means of careful plasma depletion. Before this could be done it was found that one Eck fistula dog (33-180) was unable to form enough plasma protein for *maintenance* of a normal plasma protein concentration on our basal diet used during former plasma depletion studies (4). Relatively large amounts of protein in the diet were required to keep this dog at the low normal level of plasma protein concentration.

Dog 33-180 (Eck fistula) was active and normal in appearance. This dog showed excessive thirst and diuresis. A biopsy showed some abnormalities of the liver and the clinical evidence indicates no other organ abnormality. The Eck fistula means an inefficient liver and we assume all the *evidence points to the liver* as responsible for the conspicuous difference between this dog and control animals. The dog is alive at this time and is the subject of continued study.

It is not clear why other Eck fistula dogs do not show all these abnormalities. We may argue that development of collateral circulation in some dogs may improve the circulation in the liver lobules. It is not difficult to believe that some dogs may tolerate this Eck fistula abnormality with less disturbance than others—for example, a young dog is apt to do better than an old one.

It has been recognized that *protein deficient diets* can bring about in the dog a definite decrease in the amount of plasma protein. Weech, Goettsch, and Reeves (16), using a carrot-rice-lard diet containing roughly 0.5 gm. of protein per kilo body weight per day, demonstrated in a number of dogs an average of about 2 gm. per cent drop in the plasma protein concentration in 80 days. This drop corresponds roughly to that shown by Dog 33-180 (Table 24), when twice as much

protein was being consumed. The potato-bran diet used in the present experiments, *for the length of time tested* appears to be adequate in protein for the maintenance in normal dogs of a plasma protein concentration within normal limits.

Methods

In the first experiment, three dogs with Eck fistulas and four normal dogs were studied. Later experiments were concerned with one dog (33-180) only. The basal diet consisted of boiled potatoes, canned tomatoes, Post's bran flakes, Karo, cod liver oil, cottonseed oil, and the McCollum-Simmonds (9) salt mixture. The diet was adjusted in all instances except in one normal dog to supply 0.7 to 1 gm. of protein and 80 to 100 calories per kilo of body weight per day. Plasma protein analyses were made the day before the diet was started, and frequent determinations were made during the course of the experiment on all dogs except two of the normal ones (33-11, 33-324), on which determinations were made at the beginning and at the end of the allotted time periods. Nitrogen determinations were made by the micro-Kjeldahl method with steam distillation, as modified by Goebel (13). This was checked from time to time with the macro-Kjeldahl method. The determinations on the Eck fistula dogs and on one normal dog (35-124) were carried out on oxalated plasma in duplicate and triplicate. Determinations on other dogs were carried out on citrated plasma. In all instances the dilution was corrected by appropriate factors. Albumin and globulin were determined by Howe's method as described by Peters and Van Slyke (13) using 22 per cent sodium sulfate at 37°C. Triplicate analyses were carried out, and the nitrogen determined with the micro-Kjeldahl apparatus. Urinary nitrogen was determined on the total weekly output, using measured amounts of concentrated H_2SO_4 as a preservative. For the determination of fecal nitrogen, stools were collected in concentrated H_2SO_4 . This mixture, representing the weekly output was stirred mechanically for 24 hours and aliquot samples were analyzed for nitrogen by the macro-Kjeldahl method. Plasma non-protein nitrogen determinations were done by the macro-Kjeldahl method using trichloroacetic acid for precipitation (13).

EXPERIMENTAL OBSERVATIONS

Table 21 indicates the changes in the amounts of circulating plasma protein in four normal dogs, and in three Eck fistula dogs. All of these dogs were given the potato-bran basal diet for the periods of time indicated.

In the normal dogs, there was a slight drop in the circulating plasma protein level. The drop was greatest in Dog 32-58, but the final level of this animal (6.1) is well above the lower normal limits.

The two Eck fistula dogs (34-206, 29-328) show decreases which are quite similar to those of the normal dogs. Table 21 may be misleading, in that it suggests that the drop in plasma protein concentration is uniform and gradual. This is not correct except in the case of 33-180. The most marked drop occurs during the first 2 weeks; from that time, further diminution rarely occurs. In fact some animals showed a slight increase in concentration following an initial drop.

The Eck fistula dog (33-180, Table 21) shows a totally different picture. The first period 33-180 (1) followed a long interval of kennel

TABLE 21

Effect of Potato-Bran Diet on Plasma Protein Concentration in Normal and in Eck Fistula Dogs

Dog No.	Condition	Time	Daily protein intake	Initial plasma protein level	Final plasma protein level	Initial weight	Final weight
		days	gm. per kg.	gm. per cent	gm. per cent	kg.	kg.
33-324	Normal	14	0.7	5.9	5.5	11.2	10.6
35-124	Normal	63	1.0	5.6	5.5	12.3	15.2
33-11	Normal	65	0.8	5.3	5.0	13.1	11.6
32-58	Normal	77	4.2	7.8	6.1	5.8	—
29-328	Eck fistula	42	1.0	5.2	4.9	12.3	11.8
34-206	Eck fistula	63	1.0	7.3	6.1	21.0	21.6
33-180(1)	Eck fistula	6	1.0	5.3	3.8	17.4	18.0
33-180(2)	Eck fistula	79	1.0	5.4	3.9	17.4	15.4

diet (table scraps) which is adequate in all respects. During the 6 days of this period there was a sharp fall in the plasma protein concentration from 5.3 (low normal) to 3.8 gm. per cent (close to the edema level). Obviously at this time this dog had no available reserve store of plasma protein building material. There was a slight gain in weight. The diet was supplemented with kidney to bring the plasma protein concentration up to a safe level. During this period there was no evidence of clinical abnormality. Obviously this potato-bran diet did not furnish sufficient protein for this dog to maintain its plasma protein level above the danger line.

The second period of this Eck fistula dog (Table 21, Dog 33-180 (2)) was much longer (79 days) (see also Table 24) and followed an

anemia period of 20 weeks (see Table 23) during which time the dog received a high protein diet (salmon bread, salmon, and liver (16)). It is not easy to say why this dog during this second period was able to maintain its plasma protein above the danger level for 79 days (Table 24) in contrast to the first period (Table 21). During the anemia period (Table 23) the mechanism which produces hemoglobin was rendered active and functioned efficiently, and it is possible that this general anemia reaction is concerned with the better production of plasma protein in the subsequent period. However, inefficient

TABLE 22

Effect of Diet Protein on Plasma Protein Formation (Dog 33-180 Eck Fistula)

Period	Time	Diet	Total protein intake	Plasma protein removed	Average output of plasma protein on same diet by normal dog	Average plasma protein concentration	Urinary nitrogen	A/G ratio	Weight	R.B.C. hematocrit
	days		gm. per day	gm. per day	gm. per day	gm. per cent	gm. per day		kg.	per cent
1	6	Potato-bran basal	18	0.68	3.6	5.5-3.8	2.3	1.1	17.7	38.4
2	9	Potato-bran + kidney	33	0.28	6.6	4.1	2.2	1.1	17.4	35.9
3	8	Potato-bran + kidney	25	0.26	5.0	4.3	2.4	1.1	18.1	36.5
4	7	Potato-bran + liver	33	0.23	5.9	4.3	3.5	0.9	17.7	37.1
5	7	Potato-bran + beef heart	33	0.17	5.5	4.2	2.6	0.8	18.6	38.0
6	7	Potato-bran + beef heart	50	0.20	7.6	4.3	3.5	0.9	19.3	38.9
7	7	Potato-bran + soy bean	50	0.20	10.0	4.2	5.0	1.0	19.2	38.1

kidneys have periods of improvement and a similar reaction on the part of this abnormal liver may account for this improvement in the capacity to produce plasma protein on the limited potato-bran diet. One may consider the possibility that this dog stored protein building materials during the period of heavy protein intake (anemia period, Table 23) and from this store was able to contribute to the circulating plasma proteins during the periods given in Table 24.

During this experiment (Table 22) plasma samples were taken daily. The column indicating protein removed shows the amount contained in such samples. The average plasma protein concentration in

column 7, indicates an average of the daily determinations during the given interval. Other values are determined in the same manner.

Table 22 shows the striking difference between the normal dog and the Eck fistula Dog 33-180. The Eck fistula animal cannot maintain its plasma protein concentration on the standard basal ration and we note a fall which nears the edema level (period 1). The potato-bran basal diet is continued throughout the seven periods at 18 gm. protein intake daily. Supplementing the basal diet with 15 gm. kidney protein per day (period 2) brought the plasma protein concentration level above 4.1 and the protein was then reduced giving only 7 gm. of

TABLE 23

Protein Metabolism during Bleeding and Anemia (Dog 33-180 Eck Fistula)

Period, 7 days	Diet	Protein intake	Plasma protein removed	Average plasma protein concentration	Urinary nitrogen	A/G ratio	Weight	R. B. C. hematocrit
		gm. per day	gm. per day	gm. per cent	gm. per day		kg.	per cent
1	Salmon bread-salmon-Hamburger	72	2.54	5.9	7.1	—	17.0	25.2
2	Salmon bread-salmon-Hamburger	75	2.31	6.3	8.2	—	16.9	25.6
3	Salmon bread-salmon	68	1.46	6.3	8.4	—	17.0	24.1
4	Salmon bread-liver	103	0.32	6.1	11.0	0.9	17.4	27.1
5	Salmon bread-liver	106	3.6	5.9	10.1	1.0	17.7	27.2

kidney protein in period 3. The diet was adjusted so that a plasma protein concentration was maintained at about 4.0 to 4.2 gm. per cent which is the plasma depletion level maintained in our plasmapheresis experiments. It might be assumed that this low plasma protein level would stimulate the body to form more plasma protein, as is true for the normal dog. As the food protein is increased the urinary nitrogen rises slowly and the body weight increases. The A/G ratio shows no significant change.

A normal dog depleted by plasmapheresis on these various diets would form 4 to 10 gm. of new plasma protein per day or 30 to 70 gm. per week as recorded in other experiments (10). This dog barely

maintained its plasma protein level at 4.0 gm. per cent. A normal dog shows potency ratios of 5 to 8 for these various diet mixtures while this Eck fistula dog shows a potency ratio of approximately 150. The latter figure is not accurate, as it does not take into consideration the *maintenance factor*. This maintenance factor is present of course in a normal dog but it is a relatively small factor compared with the large net plasma protein production as measured by plasmapheresis and plasma removal.

During the 20 week period while Dog 33-180 was being made anemic, the protein metabolism is of interest. 5 consecutive weeks during the middle of this period are shown in Table 23. In this period considerably greater amounts of protein were fed in order to insure maintenance of an adequate plasma protein concentration, in spite of the amount of protein being removed in the bleeding. In comparison with Table 22, it is obvious that the animal at this time was utilizing a higher protein intake in amounts sufficient to maintain a relatively high plasma protein concentration. The plasma protein concentration has risen from 4.2 gm. per cent to 6 gm. per cent in spite of the daily removal of 2 to 3 gm. of plasma protein, a significant change from this animal's previous state (Table 22). It is realized that this table does not indicate the true protein-forming ability of the animal at this time, and in order to measure it, plasmapheresis in addition to the bleeding would have to be done. This table is not included for that purpose, however, but rather to indicate that under different conditions with an extremely high protein diet, the animal was able to manufacture adequate amounts of plasma protein.

This Eck fistula dog had no reserve supply of plasma protein building materials at the start of the anemic period. The dog was put in the anemia colony and anemia produced by blood withdrawal (Dr. Robbins). The bleeding was more cautiously done than in a normal control but it became apparent that the dog did have a *considerable reserve store* of materials out of which new hemoglobin could be fabricated. This indicates that *reserve stores* which contribute to hemoglobin are independent of those which go to form plasma protein. The dog also produced a low normal output of new hemoglobin in this anemic period when fed liver. These data will be published in detail elsewhere.

Table 24 shows nine consecutive weekly intervals following the anemic period. At this time the protein intake has again been diminished to the basal level, and the amount of protein removed is only that necessary for chemical analysis. It is seen that the red blood cell percentage, as indicated by the hematocrit, slowly increased to within the original normal limits for this dog. The plasma protein

TABLE 24

Protein Metabolism during Recovery from Anemic Period—Potato-Bran Diet (Dog 33-180 Eck Fistula)

Period 7 days	Diet	Protein intake	Plasma protein removed	Average plasma protein concentration	Urinary nitrogen	A/G ratio	Weight	R.B.C. hema- tocrit
		gm. per day	gm. per day	gm. per cent	gm. per day		kg.	per cent
1	Potato-bran basal	17.5	0.35	5.3	3.28	0.8	17.4	30.9
2	Potato-bran basal	17.5	0.26	5.1	2.07	0.8	16.9	33.6
3	Potato-bran basal	17.5	0.24	4.7	2.35	0.7	16.5	34.5
4	Potato-bran basal	18.3	0.22	4.7	2.98	0.6	16.8	38.4
5	Potato-bran basal	18.3	0.22	4.9	1.98	0.5	16.5	38.8
6	Potato-bran basal	18.3	0.14	4.8	2.90	0.5	16.5	38.4
7	Potato-bran basal	18.3	0.19	4.7	2.04	0.4	16.5	40.1
8	Potato-bran basal	18.3	0.18	4.5	1.80	0.4	16.4	39.0
9	Potato-bran basal	18.3	0.12	4.3	1.83	0.3	16.1	38.8

TABLE 25

Urine Volume and Plasma Volume of Dog 33-180

Period, 7 days.....	1	2	3	4	5	6	7	8	9
Urine volume, liters.....	22.3	18.3	18.2	21.6	16.5	20.1	15.4	15.6	15.7
Plasma volume, cc.....	948	857	817	790	784	808	795	899	774

concentration gradually diminished. It is also of interest that the A/G ratio, which heretofore in this animal had been quite constant, gradually fell off from 0.8 to 0.3. Judging from other experience we were fortunate that this dog did not pick up some serious infection. A steadily falling A/G ratio usually means trouble which fortunately this dog escaped.

The diuresis and plasma volume of Dog 33-180 are shown in Table

25. This table represents the same successive periods that are shown in Table 24. A striking feature is the abnormally high urine volume with no conspicuous departure from normal noted in the plasma volume. Normal dogs of this weight will eliminate 3 to 4 liters of urine per week. It is evident that there was a diminution of water turnover in the last weeks and that the plasma volume during the whole interval diminished somewhat. This would indicate that dilution of plasma played no part in the drop in the plasma protein concentration during this period. In fact, the absolute decrease in total plasma protein would be somewhat greater than the *percentage* figures indicate, due to the slight plasma concentration. Crandall and Roberts (2) have recently reported that a number of Eck fistula dogs showed excessive water intake and urine output, and ascribe the phenomenon to interference by the Eck fistula with the "water storage function of the liver." The condition has been noted in a few Eck fistula dogs in this laboratory but has not been studied in detail.

Determinations of fecal nitrogen on the different diets showed a variation of from 15 to 18 gm. of nitrogen per week with no correlation with the amount of nitrogen in the ingested food. Plasma non-protein nitrogen determinations were done from time to time and varied between 21 and 36 mg. per cent. These figures are not of sufficient magnitude to make any appreciable difference in the figures given as plasma protein. In the estimation of plasma protein an arbitrary figure of 20 mg. was deducted from the determined total plasma nitrogen, before multiplication by 6.25.

Clinical Histories

Clinical History.—Dog 33-180. An adult male brindle bull mongrel weighing 18.5 kg. An Eck fistula was made on Dec. 28, 1933. For the next year it was used for cholesterol studies, being on a diet of table scraps (kennel diet), which was supplemented from time to time with substances high in cholesterol. For 3 months previous to the beginning of the present studies no supplements had been added to the diet. 3 months prior to the time when the present experiments were started there was evidence of a slight *intoxication* which lasted for 3 days. The blood plasma from the time of the operation had always showed a slight trace of bile pigment.

On Jan. 27, 1935, the animal was put in a metabolism cage and fasted 1 day. On the 2nd day the standard potato-bran basal ration was started. It had been planned to lower the plasma protein level by means of plasmapheresis, but during

the next few days the plasma protein level dropped so rapidly that this procedure was abandoned. It was noted that excessive amounts of urine were being excreted. Tests for sugar and albumin in the urine were done at that time, and have subsequently been repeated and these substances have never been demonstrated. 8 days from the beginning of the experiment the plasma protein concentration fell to a dangerous level (3.83), and protein in the form of cooked kidney was added to the diet. The plasma protein level rose in a few days to 4.1 to 4.2.

For the next 8 weeks various food substances whose plasma protein-regenerating qualities were known were tested on the animal. During this period its clinical condition remained excellent. For 3 months experiments were discontinued, the dog being fed during this interval the basal diet plus 150 gm. of liver daily. The next period of 20 weeks was used for hemoglobin regeneration studies, the results of which will be included in another publication. During this interval the dog was fed a relatively high protein diet (80 to 100 gm. per day), derived from salmon and salmon bread, and was bled sufficient amounts to render it anemic. Protein studies were resumed at the end of this 20 week period, at which time the plasma protein level was 5.52 and the hemoglobin percentage was 45. The clinical condition was excellent.

Potato-bran basal diet was again instituted, with addition of liver. The liver was gradually diminished over a period of 2 weeks, from which time only the basal diet was fed. This furnished 1 gm. per kilo body weight per day of vegetable protein, and 90 to 100 calories. This was continued for 10 weeks, during which time complete protein studies were done. During the latter part of this interval ulcerative lesions appeared on the buttocks but the animal remained in good general condition. Fasting was then carried out for 2 days, and at the end of this period the dog was fed the equivalent in liver of 140 gm. of protein. After a short and abrupt rise in the plasma protein level, it dropped off after 3 more weeks on the basal diet to 3.89. These data will be published separately.

At this point the dog developed considerable edema of the neck, associated with a thrombosed jugular vein, and appeared to be quite sick. The plasma was jaundiced. Large intravenous doses of dog plasma and glucose solution were given, whereupon the edema almost immediately disappeared. A high protein diet was resumed and the animal again became active and clinically normal. Within 2 weeks the plasma protein rose to 5.3 per cent. During this last experimental period of some 4 months, the weight dropped from 17.5 kg. to 15.4 kg.

Two months following this episode a *biopsy of the liver* was taken. At time of operation the liver appeared smaller than normal. It was quite yellow, and the surface was very slightly granular. Microscopic study showed moderate increase of deep red staining hyalinized connective tissue about the bile ducts and portal vessels. There was moderate round cell infiltration about some of the portal areas. In the mid-zonal regions of some liver lobules there were small foci of round cells along with a few connective tissue nuclei. The liver cells were large, pale, swollen and contained numerous tiny vacuoles. In the liver cells near the portal zones the vacuoles were much larger. By appropriate staining these large

and small vacuoles were shown to be of a fatty nature. There was no evidence of acute hepatic injury. At present the animal is in excellent condition.

Clinical History.—Dog 29-328. An Eck fistula was produced in an adult male coach mongrel on Jan. 21, 1934. The year following this operation the animal was used for hemoglobin studies and remained in excellent condition throughout this period. At the time (Feb. 27, 1935) when the present experiments were started the animal was in good condition, though moderately anemic. Potato-bran basal diet was given for a period of 44 days, during which time the plasma proteins were determined at frequent intervals (Table 21). During this period the weight dropped from 12.3 to 11.8 kg. When the experiment was terminated, the dog was in excellent condition. The red blood cell percentage, as determined by the hematocrit, had risen from 30.1 to 38.1 (Table 21).

Clinical History.—Dog 34-206. An adult male, short haired, brown mongrel, weighing 21 kg. An Eck fistula was done on June 12, 1935. The post-operative course was uneventful. Until the time when the present experiment was started, Aug. 8, 1935, the animal had been on a diet of table scraps and had shown no evidence of intoxication. Potato-bran basal diet was started on Oct. 16, 1935, and continued for 9 weeks. Frequent plasma protein determinations were made during this interval. The weight of the animal was maintained, and at the end of the period, the clinical condition was excellent. During subsequent experiments the animal died with interstitial bronchopneumonia. At autopsy the liver was small, brownish yellow. The Eck fistula was patent and the portal vein above the fistula occluded by ligature. Microscopic examination of the liver showed marked fatty change with atrophic liver cells and engorged bile canaliculi. There was hyalinization of the periportal connective tissue, but the amount of this tissue was not increased.

Clinical History.—Dog 33-11. An adult female hull mongrel, was given a potato-bran basal diet containing 0.8 gm. protein per kilo body weight over a period of 65 days. The weight during this interval was maintained, and the animal was in apparently excellent condition at the end of the period.

Clinical History.—Dog 33-324. An adult male mongrel terrier, was given potato-bran basal diet for a period of 2 weeks. The animal maintained its weight and at the end of this time was in good clinical condition.

Clinical History.—Dog 32-58. An adult female mongrel, was given potato-bran basal diet for a period of 77 days. The average daily protein intake was 4.2 gm. per kilo body weight. The dog was in good condition at the end of the period.

Clinical History.—Dog 35-124. A young mongrel collie, was given potato-bran basal diet for a period of 63 days, receiving 1 gm. per kilo body weight daily. The weight rose from 12.3 kg. to 15.2 kg. during this period and the dog was normal.

DISCUSSION

We are dealing with an Eck fistula dog which has difficulty in producing plasma protein sufficient to maintain the plasma protein

concentration above the dangerous edema level on standard low protein diets. Apparently this dog is normal in all respects but for the Eck fistula, related thirst and diuresis, and certain anatomical changes in the liver, shown by biopsy. Presumably the inability of the animal to maintain a normal plasma protein concentration lies somewhere in the chain of metabolic processes between the ingestion of food and the final elaboration of the finished product of plasma protein. It appears reasonable to assume that this dog's inadequacy in forming plasma proteins is related to an abnormal and inefficient liver.

We use the term *maintenance factor* as related to plasma proteins and indicate by that term the unknown amount of plasma protein which must be supplied day by day to take care of the "wear and tear" of the circulating protein. We assume that this maintenance factor may include certain contributions which the plasma protein may make to the body proteins, as it has been shown (14) that the fasting dog can be kept relatively close to nitrogen equilibrium by means of plasma protein given by vein. The amount of plasma protein "wear and tear" per day is probably a matter of a few grams and in this connection we note the rapid fall in plasma protein (Dog 33-180, period 1, Table 21) from 5.3 to 3.8 gm. per cent in 6 days. This amounts to 2 gm. plasma protein loss per day using the plasma volume of this dog as 800 cc. (Table 25). There is no reason to assume that the Eck fistula dog uses up more plasma protein than do normal dogs.

A point of interest in this dog is the relationship of hemoglobin formation to the decrease in plasma protein. During the interval when the plasma protein was on a decrease (Table 24), hemoglobin was being readily formed. At this time, one complex protein substance was increasing, while another protein was decreasing. Was hemoglobin being formed at the expense of plasma protein? We believe the evidence is against this hypothesis and it has been noted above that the "reserve store" of plasma protein building materials is independent of the store of hemoglobin building materials.

SUMMARY

Normal dogs and two Eck fistula dogs, receiving a daily diet containing an average of 1 gm. of vegetable protein per kilo of body weight, showed after average intervals of 7 to 9 weeks, slight decreases in amounts of circulating plasma protein (Table 21).

A third Eck fistula dog under similar circumstances was unable to maintain its plasma protein concentration above the edema level. This dog by biopsy was shown to have an abnormal liver and the evidence indicated that the other organs were normal. The animal showed active thirst and diuresis as compared with controls (Table 25).

This Eck fistula dog had less than one-tenth the capacity of the normal dog to form new plasma protein when various food proteins were added to the basal diet, and no significant quantitative differences in the relative potency of these foods (liver, kidney, heart muscle, soy bean, salmon) could be distinguished (Table 22).

It appears that the liver abnormality is responsible for this abnormal reaction. This observation gives strong support to the thesis that the liver is actively concerned with fabrication of new plasma protein.

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THE DISTRIBUTION IN THE BLOOD AND LYMPH OF PNEUMOCOCCUS TYPE III INJECTED INTRAVEN- OUSLY IN RABBITS, AND THE EFFECT OF TREATMENT WITH SPECIFIC ANTI- SERUM ON THE INFECTION OF THE LYMPH

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For several years, two of us have been interested in the passage of visible particles through the walls of blood vessels and lymphatics (1). During 1935, experiments¹ were carried out which showed that when rabbits were injected intravenously with large doses of a virulent Type III Pneumococcus, the animals developed a bacteremia, and within an hour the organisms could be demonstrated by culture to be present in the thoracic duct lymph (2). These non-motile bacteria presumably passed through the walls of blood capillaries into the tissue fluid, and then through the walls of lymph capillaries in order to reach the lymph stream.

While the thoracic duct carries lymph from all parts of the body, the greatest volume of fluid comes from the abdominal region. Judging by the protein content of liver and intestinal lymph, it is generally held that the blood capillaries in this huge region are more permeable than those in other parts of the body such, for example, as the skin and subcutaneous tissues. As a result of improvements in technique it became possible to cannulate lymphatics and collect lymph continuously from vessels in the subcutaneous tissues of the neck and occasionally the foot. It therefore seemed significant in rabbits infected with pneumococci to collect lymph draining from such areas. Should this lymph after intravenous injection of pneumococci contain the organisms, it would indicate that in the presence of a bac-

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¹ In collaboration with members of the Department of Bacteriology.

teremia the tissue spaces and lymphatics generally are invaded by pneumococci.

In addition to obtaining data on the bacterial content of the lymph from these sources, we have extended our observations on the penetration of pneumococcus antibody into the lymph following its introduction by the intravenous route. In the previous paper (2) it was shown that after the administration of rabbit or horse antipneumococcus Type III sera to infected rabbits, antibodies often fail to reach the lymph in demonstrable amounts, and even when shown to be present in considerable quantity do not lead to its sterilization. The unfortunate implications of such a situation from the standpoint of serum therapy are obvious. Our earlier experiments, however, were not extended longer than 4 hours after the injection of antiserum. Because of this it could be reasonably objected that subsequently the antibody might exert a beneficial effect on the lymphatic infection. We have, therefore, advanced the period of our observations to an interval of 18 hours following antiserum treatment. Furthermore, we have sought to eliminate, by the use of organisms in the state of maximum encapsulation, any possibility of transfer from blood to lymph in the mobile phagocytic cells.

Technique

Strains of Pneumococci.—The same rabbit virulent strain of *Pneumococcus* Type III (strain SV) (3) as previously employed was used in nearly all of the present work. Into a few animals was inoculated another strain of *Pneumococcus* Type III (strain CH) (4) which, although highly virulent for mice by the intraperitoneal route of infection, failed to kill rabbits even when given in large amounts. 16 to 20 hour rabbit blood broth cultures of these strains were usually the source of the infecting dose, but in one instance the serum of a rabbit dying after infection with strain SV was used.

Antisera.—Although several different lots of antipneumococcus Type III horse sera were injected in various experiments, that serum possessing an agglutinin titre of 1:400 was utilized in all but one of those which are reported in detail in this communication.² In one case an antiserum³ showing an agglutinin titre

² This antiserum was obtained through the courtesy of Dr. Annabelle W. Walter, of the Bureau of Laboratories of the Department of Health of the City of New York.

³ This antiserum was obtained through the courtesy of the Massachusetts State Antitoxin and Vaccine Laboratory.

of 1:20 was used. An anti-horse rahhit serum exhibiting a titre of 1:20,000 (dilution of the nntigen) against normal horse serum served to determine the quantity of horse serum present in blood and lymph, following the administration of the antipneumococcus Type III horse serum. For this purpose the ring test technique, using undiluted anti-horse rahhit serum and falling dilutions of blood serum and lymph, was adopted. Readings were taken after 2 hours at room temperature.

Blood and Lymph Cultures.—Measured amounts of blood and lymph specimens were plated undiluted and after suitable dilution in infusion broth, by the blood agar pour plate method.

Cannulation of the thoracic duct in the rahhit has been previously described (2) but slight modifications in technique have been introduced. The left external jugular vein is followed to its junction into the subclavian vein and ligated at this point. This minimizes the amount of dissection, since it is no longer found necessary to ligate the subclavian vein. All branches entering the jugular vein are carefully tied, and it is then ligated a second time about 2 cm. from its junction point into the subclavian vein. The blocked thoracic duct will then show up clearly. The venous pocket so formed is first cannulated but, if this results in bloody lymph, direct cannulation of the thoracic duct is employed, using a minimum of dissection. This, with care, is successful over 90 per cent of the time.

The cervical lymphatics are picked up easily, particularly on the left side. After the thoracic duct has been blocked by ligating the jugular vein, the cervical lymphatic which enters it will likewise become distended and can easily be recognized.

The leg lymphatics in the rahhit are extremely small and unusually delicate. In order to expose them at the ankle, 0.1 cc. of 1 per cent trypan blue must be injected subcutaneously near the toes. The lymphatics can then be picked up on either side of the saphenous vein. The binocular dissecting microscope is used both for separating the lymphatic from its connective tissue and for cannulation for which the smallest possible quartz cannula is used.

EXPERIMENTAL

To obtain information concerning the question of whether pneumococci emigrate from the blood vessels and pass into the lymphatics draining areas other than that of the abdominal region, the right cervical and right leg lymphatic ducts of a normal rabbit (weight, 1.7 kilos) were cannulated after nembutal anesthesia (1.5 cc. of 5 per cent nembutal intravenously). The deposit obtained by centrifugalization of 40 cc. of a 16 hour blood broth culture of *Pneumococcus* Type III (strain SV) was resuspended in 1 cc. of sterile broth and injected into the ear vein (4 hours after anesthesia). Samples of blood from the right jugular vein and specimens of lymph from the three sources noted above were taken from time to time and cultured. The experiment was continued for about 4 hours.

It will be seen from an inspection of the data presented in Table I that the organisms appeared promptly (within 15 to 20 minutes) in

TABLE I

Rabbit 1. Massive Intravenous Injection of Pneumococci. Recovery of Organisms in Thoracic Duct, Cervical, and Foot Lymph Almost Immediately Afterwards

Time	Organisms per cc. of				Remarks
	Blood	Thoracic duct lymph	Cervical lymph	Foot lymph	
<i>p.m.</i>					
1:10					Animal inoculated intravenously with organisms from 40 cc. of 16 hr. B.B. culture SV
1:12	5.3×10^7				
1:25		875			
1:30			2800		
1:45		1400			
1:50				3 in 1 small drop	Cannula afterwards pulled out of leg lymphatic
2:11		2300			
2:15			9600		
2:18	1.8×10^7				
2:38				1 in 2 small drops	
2:40		1500			Cannula removed from cervical lymphatic
2:47			7200		
3:05				3 in 2 small drops	
3:10		1300			
3:19	2.2×10^7				
3:40			1450		Experiment terminated
3:55					
4:05					
4:15	8.5×10^7				
4:50					
4:55	1.8×10^8				

the cervical as well as in the thoracic duct lymph. The numbers of cocci in each did not greatly differ, although on the whole those in the cervical lymph appear to be somewhat greater. In several of our

experiments definitely more organisms were cultured from neck lymph than from thoracic duct lymph. It is possible that the relatively large numbers of pneumococci in the former may be accounted for in part by the massage which is necessary in its collection. Adjacent lymph nodes as well as vessels are included in the field of massage. Even slight pressure on lymph nodes causes a rapid increase in the lymphocytes in efferent lymph. Thus it is not improbable that massage also increases the number of organisms in the lymph by forcing some of those in the nodes and tissue spaces of the neck into the lymph stream. Organisms were also found in foot lymph in the first specimen which was taken within 50 minutes after intravenous injection of culture. In the rabbit the amounts of foot lymph which can be secured at any one time are so small that accurate measurement of volume is impracticable, and therefore figures comparable with those for thoracic duct lymph and cervical lymph are not presented. We can say only that pneumococci may also quickly gain entrance to the lymphatics of the foot, although without much doubt in far smaller numbers. The results indicate that into these regions of the lymphatic bed, which are ordinarily considered less permeable to the entrance of proteins and other substances, the bacteria in question may penetrate with facility.

Since it had been found (5) that in cultures of *Pneumococcus* Type III (strain SV) of the age employed a certain percentage of the microorganisms appeared to be vulnerable to phagocytic attack and were thus removed from the circulating blood, we wished to eliminate this possible means of intracellular transfer from blood to lymph. It has been shown (5) that completely encapsulated organisms, such as occur in the blood of a rabbit dying of infection with this strain, remain in undiminished quantities in the blood, which indicates a complete resistance to ingestion by phagocytes. Following cannulation of cervical and thoracic lymph ducts, a normal rabbit was therefore injected intravenously with 13 cc. of the blood serum from a second rabbit suffering from a severe bacteremia. The bacterial content of blood and lymph specimens taken at intervals thereafter was determined. The results of this experiment are included in Table II. Here again there has been rapid passage of organisms across the

vascular lymphatic barriers in the case of both cervical and thoracic duct lymph, although the possibility of movement of cocci in phagocytes has been substantially ruled out. As further evidence for the failure of phagocytic carriage to account for these findings we may mention experiments not here reported in detail in which large numbers of *Pneumococcus* Type III (strain CH) were injected. These organisms had already largely lost their capsules during the course of

TABLE II

Rabbit 2. Intravenous Injection of Heavily Infected Serum from a Second Rabbit Moribund as a Result of Pneumococcus Infection. Phagocytosis as a Means of Transfer from Blood Thus Eliminated

Time	Organisms per cc. of			Remarks
	Blood	Thoracic duct lymph	Cervical lymph	
<i>p.m.</i>				
12:30				13.0 cc. of infected rabbit serum injected intravenously
12:32		0		
12:35	1×10^8			
12:45		20		
12:47			250	
12:55	8.2×10^7			
1:27		20,000		
1:32			6000	
2:35	3.4×10^8			
2:40				Thoracic duct lymph suddenly became bloody
2:45			9000	
3:30	3.7×10^8			
3:45			9000	
4:00				Experiment terminated

growth and in consequence the great majority were readily removed from the blood stream, presumably by phagocytic cells (5). In this condition these cocci did not appear at all in the cervical lymph, while in that from the thoracic duct, although most specimens were sterile, in a few an extremely small number of pneumococci were detected. These observations show that when massive phagocytosis of the organisms takes place the lymph is either not invaded at all or only to a minimal degree.

Having established the fact that the pneumococci migrate into the cervical and foot lymph as well as the thoracic duct lymph, and that this migration is not mediated through the agency of phagocytic ingestion, we next proceeded to reinvestigate the effect of antiserum on the organisms in these sites, partly because lymph derived from different regions was now available and partly to study the course of events during periods farther removed from the time of inauguration of serum treatment than those previously recorded. Details of two typical experiments on this point are given in Tables III and IV.

The animal which yielded the data summarized in Table III was inoculated with a small dose of pneumococci 23½ hours before the administration of 15 cc. of antipneumococcus Type III horse serum¹ (agglutinin titre 1:20). The organisms in the blood, which were present in moderate numbers when the serum was given, were temporarily reduced within a brief period to zero, although subsequently they reappeared. In this case the initial number of pneumococci in the thoracic duct lymph was comparatively small, and this may have undergone a transient reduction following antiserum but soon increased. Cervical lymph on the other hand contained relatively large quantities of cocci. These also underwent some diminution in the course of the experiment, although sterility was not obtained within the 7 hours after the injection of antiserum. Two samples of leg lymph proved sterile on culture.

The protein content of cervical and thoracic duct lymph was similar but the cell counts were uniformly higher in the cervical specimens. In both types of lymph the cells were almost entirely lymphocytes and the presence of pneumococci did not alter the differential counts. On the average the large mononuclears were found to comprise from 2 to 6 per cent of the total number of cells while polymorphonuclear leucocytes were absent.

These observations, then, indicate that within the 7 hours following antiserum administration the lymph fails to become free of pneumococci. It should be noted, however, that the antiserum contained a rather low titre of antibody as measured by its agglutinating capacity. This might account for its failure to lead to the elimination of the cocci in the lymph. In the experiments which follow, similar results were secured although an antiserum exhibiting an agglutinin titre far greater was used.

TABLE III

Rabbit 3. Intravenous Infection Followed by Antipneumococcus Type III Horse Serum after 23½ Hours

Time	Organisms per cc. of				General data and remarks
	Blood	Thoracic duct lymph	Cervical lymph	Foot lymph	
Mar. 17, 1936 a.m. 11:00					Intravenous inoculation of 0.2 cc. of 16 hr. B.B. culture of SV. Rectal temperature 103.0°F.
Mar. 18, 1936 a.m. 9:10					1.2 cc. of 5 per cent nembutal intravenously Rectal temperature 105.7°F.
10:05					
10:25	2400				15 cc. antipneumococcus Type III horse serum intravenously (H536, N. Y.)
10:33					
10:45	50				Leucocytes in blood 2600 per c.mm.
11:50					
p.m. 12:30					Protein in thoracic duct lymph 3.55 per cent
12:37					Erythrocytes in thoracic duct lymph 500 per c.mm. Leucocytes in blood 3200 per c.mm. Rectal temperature 106°F.
12:40					Protein in cervical lymph 3.56 per cent
12:50					Leucocytes in thoracic duct lymph 37,200 per c.mm.
1:00					Leucocytes in cervical lymph 55,400 per c.mm.
1:15	0				Erythrocytes in cervical lymph 500 per c.mm.
1:20		7			
1:35			1200		
1:55					Protein in leg lymph 1.23 per cent
2:25					Leucocytes in thoracic duct lymph 34,400 per c.mm. Erythrocytes in thoracic duct lymph 500 per c.mm.
2:45					Leucocytes in blood 4600 per c.mm.

TABLE III—*Concluded*

Time	Organisms per cc. of				General data and remarks
	Blood	Thoracic duct lymph	Cervical lymph	Foot lymph	
p m.					
2:55					Protein in cervical lymph 3.63 per cent
2:58					Protein in thoracic duct lymph 3.33 per cent. Leucocytes in cervical lymph 129,800 per c.mm. Erythrocytes in cervical lymph 3400 per c.mm.
3:05					Rectal temperature 106.1°F.
3:12				0	
3:25			2000		
3:35		0			
4:05	0				
4:20	120				Thoracic duct lymph 49,400 leucocytes per c.mm., and 1200 erythrocytes per c.mm.
4:22				0	
4:40		30			
4:47			900		
4:50					Thoracic duct lymph, leucocytes 63,400 per c.mm. and erythrocytes 1200 per c.mm.
4:55					Rectal temperature 108°F.
4:56	77				Leucocytes in blood 3400 per c.mm.
5:00					Cervical lymph, leucocytes 129,400 per c.mm. and erythrocytes 9600 per c.mm.
5:12			250		
5:16				0	
5:19		10			
5:25					Experiment terminated

The data assembled in Table IV were obtained in the case of a rabbit which received two doses of antiserum, at 33 hours and again at 50 hours, following intravenous inoculation of a small dose of strain SV culture. Examination of the results shows that the first dose of antiserum reduced the organisms present in the circulating blood, but 16 hours later, at the beginning of the observations following cannulation of the lymphatics, organisms were detected in the blood

TABLE IV

Rabbit 4. Intravenous Infection with Small Dose of Pneumococci, Followed 33 Hours Later by Intravenous Administration of Antipneumococcus Type III Horse Serum. Recovery of Organisms in Thoracic Duct and Cervical Lymph, Both before and after a Second Injection of Antiserum (17 Hours Subsequent to First Serum Treatment). Titration of Blood and Lymph for Presence of Antibodies and Also Horse Serum Vehicle

Time	Organisms per cc. of			Agglutinin titre vs. Type III Pneumococcus	Precipitinogen titre horse serum vs. anti-horse rabbit serum	General data and remarks
	Blood	Thoracic duct lymph	Cervical lymph			
May 4, 1936 p.m.						
2:00	Animal inoculated intravenously with 0.05 cc. of 21 hr. blood broth culture of SV					Rectal temperature before inoculation 102.7°F. Leucocytes in blood before inoculation 6500 per c.mm. Rectal temperature 103.5°F.
5:00						
May 5, 1936 p.m.						
3:15						
10:40	5300			0 (blood)	0 (blood)	Rectal temperature 105.8°F.
11:00						Rectal temperature 105.7°F. Intravenous injection of 7.5 cc. antipneumococcus horse serum (New York)
11:30	2000			1:50 (blood)	1:600 (blood)	1.6 cc. nembital intravenously Leucocytes in blood 4800 per c.mm.
May 6, 1936 a.m.						
9:15						
9:45						
p.m.						
12:30						Rectal temperature 102°F. Thoracic duct lymph, 25,400 leucocytes per c.mm., 6200 erythrocytes per c.mm.
12:55						

1:30	2500	1.1×10^8	180 per small drop	0 (thoracic duct lymph) 0 (blood)	1:200 (thoracic duct lymph) 1:300 (blood)	Thoracic duct lymph, protein 3.74 per cent Leucocytes in blood, 4800 per c.mm.
2:30						
2:40						
2:50						Thoracic duct lymph, 70,400 leucocytes per c.mm., 1200 erythrocytes per c.mm.
3:10	2500	1.9×10^8	∞ colonies per drop	0 (thoracic duct lymph) 0 (blood)	1:200 (thoracic duct lymph) 1:300 (blood)	Cervical lymph, 137,200 leucocytes per c.mm., no erythrocytes seen Rectal temperature 104.5°F.
3:20						
3:30						
3:40						
3:45	2500	1.9×10^8	∞ colonies per drop	0 (thoracic duct lymph) 0 (cervical lymph) 1:3 (blood)	1:300 (thoracic duct lymph) 1:600 (cervical lymph) 1:600 (blood)	Intravenous inoculation of 5 cc. of antipneumococcus horse serum (New York)
3:55						
3:57						
4:00						
4:18	100 or less	1.2×10^8	6.7×10^8	0 (blood) 0 (thoracic duct lymph)	1:800 (blood) 1:500 (thoracic duct lymph)	Thoracic duct lymph, 52,800 leucocytes per c.mm., 1400 erythrocytes per c.mm.
4:25						
4:30						Blood, leucocytes 1200 per c.mm.
4:55						
5:03	14,500	1.2×10^8	∞ colonies per 0.05 cc.	0 (blood) 0 (thoracic duct lymph)	1:800 (blood) 1:500 (thoracic duct lymph)	Cervical lymph, 81,800 leucocytes per c.mm., 400 erythrocytes per c.mm.
5:05						
5:07						
5:10						
5:11						

TABLE V

Rabbit 5. Intravenous Infection with Small Dose of Pneumococci, Followed after 24 Hours by Intraperitoneal Administration of Antipneumococcus Type III Horse Serum. Titration of Antibody in Blood and Lymph as well as Determination of the Presence of Organisms in These Fluids

Time	Organisms per cc. of				Agglutinin titre cc. Type III Pneumococcus	Precipitinogen titre horse serum vs. anti-horse rabbit serum	General data and remarks
	Blood	Thoracic duct lymph	Cervi- cal lymph	Foot lymph			
May 18, 1936 p.m. 4:55							
May 19, 1936 a.m.	Animal inoculated intravenously with 0.1 cc. of 23 hour blood broth cul- ture of SV						
11:00	3.3×10^4						Rectal temperature 103.8°F.
p.m. 4:55	9.7×10^4				0 (blood)	0 (blood)	Rectal temperature 105.6°F.
5:05					1:3 (blood)	1:200 (blood)	10 cc. antipneumococcus Type III horse serum (New York), intraperitoneally
6:05	180				1:1 (thoracic duct lymph)	1:300 (thoracic duct lymph)	Rectal temperature 104.6°F.
May 20, 1936 p.m. 12:20		1720					
12:36							Blood, 10,600 leucocytes per c.mm.
12:55							Thoracic duct lymph, 50,800 leucocytes per c.mm., 1200 erythrocytes per c.mm.
1:00							

1:10					1:300 (thoracic duct lymph)	
1:11	0			0		Blood, 7400 leucocytes per c.mm.
3:00						
3:40						
3:54						
4:10	530				1:400 (blood)	Thoracic duct lymph, 67,400 leucocytes and 400 erythrocytes per c.mm.
4:15						
4:20						
4:45					1:300 (thoracic duct lymph)	Cervical lymph, 139,000 leucocytes and 3600 erythrocytes per c.mm.
4:50						Blood, 8600 leucocytes per c.mm.
4:55						Thoracic duct lymph, 32,400 leucocytes and 3400 erythrocytes per c.mm.
5:05				7060		
5:10						
5:25	530			1.9×10^6		Experiment terminated

in numbers practically equivalent to those observed shortly after antiserum was first introduced. A finding which calls for particular emphasis is the fact that a definitely greater number of cocci were present at this time in the thoracic duct lymph and probably also in the cervical lymph, than were cultivated from the blood. 17 hours after the first dose of antiserum a second injection of 5 cc. was given. This resulted in a reduction of approximately 95 per cent of the organisms in the blood. The larger numbers in the thoracic and cervical lymph showed no evidence of decrease but continued to rise. This may serve to explain the subsequent large increase in the bacterial count of the blood at the close of the experiment, which may well have resulted from the influx of organisms from other intact lymphatics, such as the remaining cervical duct which had not been cannulated.

Titration to determine the presence of agglutinins in blood serum and lymph, indicate that the considerable amount of antibody in the serum immediately after the first injection of antiserum had completely disappeared by the time the operation on the following day was completed. A small amount of antibody in the blood serum was found in the sample taken just after the second antiserum injection, but this was speedily exhausted. No agglutinating antibody could be detected in any sample of lymph. That the horse serum vehicle had gained entrance to the lymphatic system in abundance was readily demonstrated by means of precipitin tests with anti-horse rabbit serum. The failure to discover antibody in the lymph and its rapid disappearance from the blood, can be attributed without much doubt to the large numbers of organisms present, as well as their soluble antigenic products which would speedily neutralize considerable quantities of agglutinin. In general the results clearly indicate that, even at intervals of many hours following antiserum treatment, the pneumococci may be found in large numbers in the lymph, where a second intravenous injection of antibody fails to reduce them.

The experiment recorded in Table V serves to confirm these findings. In addition it shows that, although demonstrable antibody may be observed in the lymph after 24 hours following intraperitoneal injection of antiserum in the case where the number of cocci

in the lymph is moderate, nevertheless antibody at least in this quantity is insufficient to effect a permanent reduction or to inhibit their multiplication.

DISCUSSION

In this paper are presented the results of experiments which supplement and extend those reported in 1935. They make it quite apparent that, during the course of the severe bacteremia following intravenous infection of the rabbit with virulent *Pneumococcus* Type III, the bacteria readily pass through the vascular walls, migrate through the tissue spaces, and enter lymphatic vessels. This emigration from the blood stream can take place not alone through the capillaries of the highly permeable abdominal region, since during this investigation it has been found that the organisms are present in both the lymph of the cervical duct and of the lymphatic vessels of the foot, as well as in that of the thoracic duct. Thus one may fairly conclude that hematogenous infection of the lymphatics may occur at almost any site.

Although, in our former experiments, we felt that it was unlikely that the conveyance of the pneumococci from blood to lymph was effected by phagocytic cells which had ingested the organisms but had failed to destroy them, there was nevertheless the possibility that entrance to the lymphatics was gained by this means. Through the use of completely encapsulated organisms (animal), it has been shown that these readily infect the lymph although entirely resistant to phagocytic attack, while pneumococci in the decapsulated state which are easily taken up by phagocytes enter the lymph in minimal numbers or not at all.

Further information concerning the effect of the intravenous administration of antiserum on the organisms in the lymphatics has been acquired which indicates that even after many hours and repeated doses the antiserum remains without decisive action in reducing the severity of the lymphatic infection. This failure may depend on a number of factors which can be surmised from the data recorded in this and the previous paper, as well as from the bacteriological, immunological, and physiological facts concerning the mode of resistance of the body against infection with this organism and

the passage of proteins across the vascular lymphatic barriers. Since it is well known that antibody alone is not sufficient to bring about the destruction of the pneumococcus, but that subsequent to sensitization with the immune substance ingestion by the phagocytic cell with attendant intracellular digestion must follow, it is obvious that unless such cells are present in adequate numbers in the lymphatic system, the antibody in concentrations however great will not of itself lead to the elimination of the cocci. In the course of our work, cytological studies on blood and lymph have shown that in the blood the leucocyte count tended to be low, with a large proportion of the cells represented by lymphocytes. In the thoracic duct lymph, the increasing content of pneumococci throughout various experiments seems to have caused no marked changes from the cell counts and relationships which have been noted in normal rabbits. In uninfected animals variations from 11,300 to 44,960 per c. mm. have been reported (6),⁴ and differential counts have indicated that about 88 per cent are small lymphocytes. In one of our experiments on an animal with heavily infected thoracic duct lymph, four differential counts showed 92, 95, 88, and 95 per cent small lymphocytes. The remaining cells were large lymphocytes. No polymorphonuclear leucocytes were found and large monocytes only after prolonged search. Thus it would appear that the only cells available for phagocytosis are tissue cell phagocytes of the lymph nodes and it is probable that these are too few to cope with the heavy infection which exists under the conditions of our experiments. The absence, then, of an effective cellular defence in the lymph may well represent one factor in the failure of antiserum to function effectively. A second is suggested by the rapid fall in titre of antibody following injection of antiserum into the blood stream of the infected rabbit, which was observed in many cases. This points to the neutralization of a large proportion of antibody by the cocci and their soluble antigenic products. The same relative reduction would take place in the quantity of any antibody reaching the infected lymph. Evidently it is necessary to introduce very large amounts of antibody to overcome this effect even in the blood stream. It would appear to be even more difficult to attain sufficient concentration of im-

⁴ Drinker and Field (6), page 122.

mune substances in the lymph because of the more or less fixed ratio which exists between the concentrations of blood protein and lymph protein, whether this be homologous or foreign. This ratio is about 2 to 1 for the homologous protein of blood and lymph in rabbits. Various workers (6)⁶ have shown that the antibody content of lymph from various sources compared with that of the blood follows closely the ratios for the normal protein content of the two fluids. Presumably by increasing the vascular permeability following the administration of antiserum, antibody in larger quantity might be delivered to the lymphatic system. Available measures for increasing vascular permeability nevertheless are generally so radical that their use subsequent to antiserum treatment seems inadvisable.

SUMMARY

Experiments are described which show that in rabbits infected intravenously with virulent Type III pneumococci, these organisms are found not only in the thoracic duct lymph, as previously reported, but also in lymph from the cervical and leg lymphatics. The non-motile bacteria must have crossed both vascular and lymphatic endothelium in reaching the lymph. Intracellular transportation by phagocytes is apparently not the means by which this is effected.

The intravenous and intraperitoneal injection of large amounts of homologous type-specific antibody fails even after many hours to terminate or permanently reduce the pneumococcal infection of the lymph.

The failure of antiserum to sterilize the lymph is discussed.

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⁶ Drinker and Field (6), page 127.

A QUANTITATIVE STUDY OF THE CROSS REACTION OF TYPES III AND VIII PNEUMOCOCCI IN HORSE AND RABBIT ANTISERA*

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Atypical Type III strains of pneumococci were first isolated by Sugg, Gaspari, Fleming, and Neill (1). These strains gave cross reactions with Type III antisera, but failed to remove all of the antibody. Cooper, Edwards, and Rosenstein (2) classified these atypical strains as Type VIII and found that incomplete crossing occurred in antisera to both types. Paralleling the immunological relationships is a close similarity of the type specific polysaccharides. Thus the Type III substance is a polyglucuronoglucose (3), while Goebel has found the Type VIII substance to contain the same aldobionic acid (glucuronoglucose and glucose in addition (4).

The development of quantitative chemical methods for the micro estimation of precipitins (5, 6) and agglutinins (7) offered an accurate means of studying the extent and nature of this cross reaction. In the course of this study the specific polysaccharide of Type VIII pneumococcus was prepared by the method recently published from this laboratory (8).

EXPERIMENTAL

Materials.—Type III and Type VIII horse and rabbit antipneumococcus sera were used in this investigation.¹ Sera H 636, H 607, R 3.50₂, and R 3.49₂ were

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** Rockefeller Foundation Fellow, 1934-1936.

¹ The horse sera were kindly supplied by Dr. William H. Park and Miss Georgia Cooper of the New York City Department of Health Laboratories, and the Type VIII rabbit sera by Drs. Goodner and Horsfall of the Hospital of The Rockefeller Institute for Medical Research.

absorbed with C substance and R protein derived from a Type I strain of pneumococcus. The bacterial suspensions used for the agglutinin determinations were prepared as described in Reference 7. The Type III and Type VIII pneumococcus polysaccharides (hereafter referred to as S III and S VIII) were isolated without the use of heat, strong acid, or alkali (8).

In the preparation of S VIII,² it was found that after the initial alcohol precipitation of the material from the vacuum concentrate of 10.5 liters of a 16 hour broth culture, it was necessary to use only 0.5 volume of alcohol as precipitant in the presence of sufficient sodium acetate and acetic acid. The yield was 0.61 gm. of the dried sodium salt. Its properties are given below and are in essential agreement with the portions of the data given by Brown (9) and by Goebel (4). From the high viscosity, it is to be presumed that the new preparation is in a less degraded form than the earlier products in which heat was used (*cf.* 8). The S VIII contained a small, but serologically demonstrable, amount of C substance.

Properties of the Type VIII Pneumococcus Polysaccharide

Ash as Na	N*	$[\alpha]_D^{26}$	Neutral equivalent†	Acetyl*	Uronic anhydride*	Reducing sugar on hydrolysis*	η_{rel} 0.10% solution in 0.9% NaCl	η_{rel} 0.20% solution in H ₂ O
per cent	per cent	degrees		per cent	per cent	per cent		
3.1	0.2	+123	720†	0.5	27†	87	2.50	16.6

* Calculated to the ash-free basis.

† Calculated from Na content.

‡ Calculated for $[(C_6H_{10}O_5)_3(C_6H_8O_6)]_x$: Neutral equivalent, 662; Uronic anhydride, 26.6%.

The precipitin determinations were carried out by addition of known amounts of specific polysaccharide to accurately measured quantities of serum and determination of the specifically precipitable nitrogen after 48 hours at 0° in the washed precipitates by the micro Kjeldahl method (5, 6). Agglutinin determinations were made similarly (7), using measured volumes of washed, heat-killed pneumococcus suspension and estimating the increase in nitrogen content. All determinations were run, in duplicate, at 0° for 48 hours to remove antibody as completely as possible. The results are summarized in Table I. Under the columns headed Precipitin N and Agglutinin N are listed the values for the maximum amount of antibody found, using the specific polysaccharide and the bacterial suspension, respectively. To estimate the group specific antibody present, agglutinin determinations were carried out with a pneumococcus I R (Dawson S) suspension. In the last column are tabulated the sums of the agglutinin found, using the heterologous strain first, followed by determination of the remaining

² The Type VIII pneumococcus strain was kindly supplied by Dr. O. T. Avery of the Hospital of The Rockefeller Institute.

antibody on an aliquot portion of the supernatant with a suspension of the homologous type. Since this procedure involved up to four successive sets of analyses on the same solution, agreement with the directly determined homologous agglutinin N was not always perfect.

In Table II are given data on the addition of increasing amounts of S VIII and S III to Type VIII horse antiserum, H 644. Before setting up these experi-

TABLE I

Quantitative Agglutinin and Precipitin Determinations on 1 Ml. of Horse (H) and Rabbit (R) Antisera to Pneumococcus III and VIII

Serum No.	Serum type	Antipneumococcus III content		Antipneumococcus VIII content		Anti-I R	Heterologous agglutinin N + homologous agglutinin N
		Precipitin N	Agglutinin N	Precipitin N	Agglutinin N	Agglutinin N	
		mg.	mg.	mg.	mg.	mg.	mg.
H607, 1:1	III	0.68	0.75	0.11	0.22	0.11	0.79
H636	VIII	0.33	0.40	0.99	1.20	0.11	1.15
H644, 2:5*	VIII	0.55	0.65	1.46	1.42	0.35	1.30
R3.50 ₂	III	2.38	2.43	0.00	0.09	0.03	
R3.49 ₂	III	0.76	0.86	0.04	0.23	0.15†	
R7.18	VIII	0.06	0.25	>1.36	1.59†	0.10	1.56
R7.19	VIII	0.00	0.20	0.84	1.21‡	0.11	1.10
		Antipneumococcus I content					
R3.70, 1:2.5	I	0.43§	0.45				

* This serum still contained anti-C. Since the S VIII contained C substance, the relatively high precipitin N value is accounted for.

† The supernatant, now free from anti-C, gave a definite precipitate with S VIII.

‡ The supernatants from the Type III precipitin determinations were used. 0.06 was therefore added to the agglutinin found in the R 7.18 serum. Owing to the small quantities of the Type VIII rabbit sera available, analytical samples of 0.5 ml. were used. The usual error was therefore doubled.

§ The supernatant, set up with pneumococcus I S suspension, yielded no more agglutinin N.

ments, 16 ml. of the serum were diluted to 40 ml. and absorbed with C substance, so that only precipitin for the type specific carbohydrates would be measured. In order to ascertain whether or not precipitation of the Type VIII serum with S III resulted in a change in the quantitative relationships of the residual antibody, the supernatants from the S III precipitations were combined, treated with an excess of S III, and again centrifuged. Portions of the supernatant were then set up with S VIII as in Table II. Calculations were made from the data as in

TABLE II
Antibody N Precipitated from Type VIII Antiserum H 644 by Varying Amounts of S VIII and S III

Amount specific polysaccharide used	Antibody N precipitated by S VIII serum dilution	Ratio antibody N precipitated to S VIII used	Tests on supernatants	Antibody N precipitated by S III from same serum dilution	Ratio antibody N precipitated to S III used	Tests on supernatants	Antibody N precipitated by S VIII from 1.5 ml. serum dilution freed from cross reacting antibody	Ratio antibody N precipitated to S VIII used	Tests on supernatants
mg.	mg.			mg.			mg.		
0.020							0.362	18.1	Excess A
0.030							0.508	16.7	" "
0.050	0.820	16.4	Excess A*	0.400	13.6	Excess A†	0.630	12.6	" "
0.059									
0.075	1.036	13.8	" "	0.502	8.5	" "			
0.089							0.676	9.0	No A or S
0.100	1.128	11.3	Trace A (?)	0.578	6.5	" "			
0.118							0.678	6.8	" "
0.150	1.116	7.4	No A or S	0.654	(5.7)	Trace S			
0.177							0.670		Excess S
0.200	1.110		Excess S	0.736		Excess S			
0.236						" S	0.670		" "
Equation:									
							mg. N pptd. = 21.4 S - 101 S ²		
							S max. = 0.106†		
							A max. = 1.136†		

The S III-anti-S VIII reaction is inhibited by high concentrations of S III.

* A = antibody.

† In addition there was no precipitate with H 644 serum or C-absorbed pneumococcus III rabbit antiserum, showing absence of S III in supernatant.

‡ Values calculated from the equation.

(10) and it was found that the general equation there derived held for the reaction between S VIII and its homologous antibody. The data are also plotted in Fig. 1 as recalculated to 1.00 mg. of antibody N so that the different experiments may be compared. The equations used are:

$$\text{mg. antibody N pptd.} = 2RS - \frac{R^2}{A} S^2, \text{ and}$$

the linear relation,

$$\frac{\text{mg. antibody N pptd.}}{S} = 2R - \frac{R^2}{A} S,$$

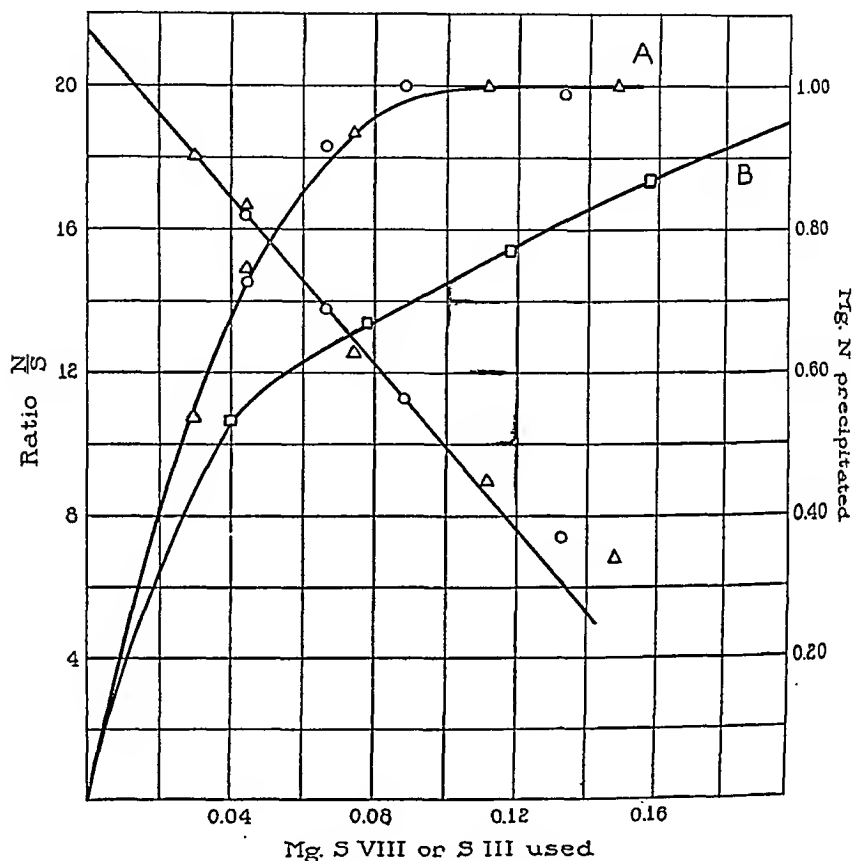
in which R is the ratio of antibody N to S at a reference point in the equivalence zone and A is the amount of antibody precipitated at that point. It will be noted that the cross reaction fails to follow these equations.

DISCUSSION

The quantitative study of the homologous and cross reactions of Types III and VIII pneumococcus and their respective antisera derived from the horse and from the rabbit has revealed several points of interest. As will be seen from Table I, homologous precipitin is, in general, approximately equal to homologous anticarbohydrate agglutinin both in the horse and rabbit antisera, if the antibody reactive with pneumococcus I R be deducted. This not only furnishes additional instances (*cf.* 11) of the quantitative correspondence of anticarbohydrate precipitin and agglutinin but indicates also that the specific polysaccharides used as reagents for precipitin were isolated (8) in a form in which their reactivity differs little from their behavior toward antisera when present in or on the bacterial cell. This is the more significant since S I prepared by Chow's recent modification of the above method employing trichloroacetic acid (12) is stated by this worker to precipitate only 35 per cent of the antibody in a Type I antipneumococcus rabbit serum. S I prepared according to Reference 8 precipitates practically all of the anticarbohydrate in rabbit serum as well as in horse serum (11), as shown in the last line of Table I.

As for the cross reactions, the results shown in Table I are somewhat complicated by the presence of group specific antibody even in the sera which had been absorbed with C substance and R protein.

The amount of this antibody is shown in Table I under Anti-R agglutinin. In spite of this it is evident that a larger proportion of



TEXT-FIG. 1. Reaction of S VIII and S III in antipneumococcus VIII horse serum, absorbed with C substance.

O, S VIII-anti-S VIII reaction.

□, S III-anti-S VIII reaction.

Δ, S VIII-anti-S VIII reaction after removal of antibody precipitated by excess S III.

All reactions calculated to 1.00 mg. of total antibody N.

the anticarbohydrate in the horse sera was cross reactive in both directions than in the rabbit sera, and that the two anti-VIII horse sera tested contained a higher proportion of cross reacting anticarbo-

hydrate than did the anti-III serum. Of the rabbit sera, one Type III and one Type VIII serum failed to show crossing with the heterologous specific polysaccharide, a failure which did not depend upon the total antibody present, since the non-reactive III serum (R 3.50₂) contained three times as much antibody as did the cross reacting III serum (R 3.49₂). Neill and his associates (1) also noted variations in the cross reactivity of both horse and rabbit antisera which did not depend on the homologous antibody titer.

A possible practical application of the data summarized in Table I would be the absorption of diagnostic Type III and Type VIII antisera with Type VIII and Type III S (Dawson M) pneumococci, respectively, in order to eliminate crossing due to S III, S VIII, and group specific components.

As shown in Table II and Fig. 1 (curve A and the line) the precipitin reaction between the specific polysaccharide of Type VIII pneumococcus (S VIII) and its homologous antibody may be quantitatively expressed, in the region of excess antibody, by an equation of the same form as that derived for the Type III reaction (10), and would therefore appear to be governed by the same mechanism. The cross reaction between the Type III pneumococcus polysaccharide (S III) and Type VIII antibody, however, appears to be of a different nature, and the curve (B) obtained by plotting the amount of antibody nitrogen precipitated against S III added is of a different form. It will be noted (Table II) that a large excess of S III is required in order to precipitate all of the cross reacting antibody. While this recalls in some measure the behavior of crystalline egg albumin in the cross reaction with the antibodies to R-salt-azo-biphenyl-azo egg albumin (13), the S III-anti-S VIII compounds formed gave no evidence of the dissociation which was so marked in the egg albumin-anti-dye cross reaction. This was shown by the failure of the supernatants from the S III-anti-S VIII precipitates in the region of excess antibody to react with either Type III or VIII antiserum which had been absorbed with C substance. An additional difference is the inhibition of precipitation caused by a large excess of S III. The two cross reactions of which a quantitative study has been made are therefore of quite different character, and this is also brought out by the relatively small proportion of cross reacting antibody in the present

instance. It is also striking that the 70 per cent of residual, strictly type specific anti-S VIII, after removal of the antibody precipitated by S III, followed exactly the same equation, when calculated to the same antibody content (see Fig. 1) as did the whole antibody. This would indicate that no fractionation had occurred with respect to reactivity with S VIII, and that the antibody removed by S III had the same quantitative relationship to S VIII as did the portion which did not react with S III.

It is believed that the quantitative data accumulated in the present study also throw light on certain of the chemical and immunobiological factors concerned in pneumococcus type specificity and the cross reaction between types. It will be noted from Table I that the rabbit antisera studied contain little or no cross reacting antibody, although their content of antibody reactive with the homologous polysaccharide is as large as that of the horse sera. Indeed, a rabbit Type III antiserum, 3.50₂, with the unusually high titer of 2.4 mg. of homologous anticarbohydrate nitrogen per ml., failed entirely to precipitate S VIII. Closely related as are S III and S VIII chemically in their content of the same structural unit (4), the pneumococcus antigens of which these polysaccharides form the determinants of specificity are capable of eliciting in the rabbit abundant production of strictly type specific antibody. Although this antibody reacts quantitatively as completely with the homologous specific polysaccharide as it does with the entire antigen (as, for example, heat-killed pneumococci of the same type), it is inert, or nearly so, toward the closely related polysaccharide or antigen of the other type. This, it is believed, indicates that the true type specific antibody is directed toward multiples of the characteristic chemical structural unit of the homologous specific polysaccharide. The implications of this interpretation will be discussed in greater detail when an experimental test of its validity, now under way, has been completed. It would appear, however, that the common aldobionic acid component of the two polysaccharides, a glucuronoglucose, is concerned in the true type specific response only as part of a larger, type characteristic structural unit.

As for the cross reaction, there seems no reason to alter the view of Avery, Heidelberger, and Goebel (14) that cross reacting polysaccharides "contain in a portion of the complex molecule the same or

a closely similar configuration of atoms." It would appear premature to attempt to localize even the cross reactivity in a more definite portion of the common configuration. Goebel, for example, has emphasized the importance of the uronic acid portion of the aldobionic acid (4, 15), but Avery and Goebel (16) have shown that the rotation of a single carbon atom through an angle of 180° , as in glucose and galactose, produced as profound a change in specificity as did the substitution of a terminal —COOH group, as in glucuronic acid, for $\text{—CH}_2\text{OH}$, as in glucose (15). Moreover, precipitation of an antipneumococcus Type III horse serum with an artificial glucuronic acid antigen (15) must either have removed antibody unrelated to the cross reaction with S VIII, or else removed a quantitatively insignificant amount of antibody, since it failed to reduce the qualitative cross reaction titer to S VIII.

SUMMARY

1. A preparation of the specific polysaccharide of Type VIII pneumococcus is described in which the use of heat, strong acid, and alkali were avoided.

2. Quantitative estimations are given of the homologous and cross reacting precipitin and agglutinin in Types III and VIII antisera produced in the rabbit and in the horse. Quantitative data are also given on the mechanism of the Type VIII precipitin reaction and the cross reaction between the Type III polysaccharide and Type VIII antipneumococcus horse serum.

3. The significance of the data is discussed.

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REINFECTION (SECOND ATTACK) IN EXPERIMENTAL POLIOMYELITIS

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The phenomena of immunity in poliomyelitis have been the subject of increased interest and experimental inquiry in the last few years. To a certain extent the renewed interest owes its origin to the efforts made recently to immunize children in mass against the disease (1). The undertaking, now discontinued, was based on two observations made early in the experimental study of the malady, (a) that monkeys which have recovered from an attack tended to resist reinoculation with the virus and (b) that animals given a number of subinfective doses developed humoral neutralizing antibodies (2). The existence of the immunity and the presence of the antibodies were so correlated as to make it appear that the one condition depended on the other.

As experience has grown it has become apparent that the two immune states, one based on recovery from an attack of the disease and the other a symptomless reaction to virus injections, are not strictly identical. They do agree in that under both sets of conditions humoral antibodies usually appear; they differ in that symptomless immunization is less protective against reinoculation than is the state of resistance which develops upon an active or symptomatic infection.

On the other hand, that reinfection, called second attack, does occur after recovery from frank poliomyelitis is established for children and for monkeys (3). The phenomenon of reinfection has been given little attention and so far has been dealt with only in connection with the epidemic disease in man concerning whom some dozen cases have been reported in the literature. The problem of reinfection can be more thoroughly studied in the monkey, and this paper will be devoted to the presentation of the results of such a study which has been carried on during the past several years as material for it became available.

It is well known that experimental poliomyelitis is far more severe

and fatal in the monkey than the natural disease in man. Although the monkey is not a natural host, it can be infected readily with the virus by means which closely simulate the mode of infection now recognized as occurring ordinarily in man. When the paralysis in monkeys is extensive, death usually results; but every so often one of these animals may be nursed back to recovery which, as in man, is sometimes complete and sometimes attended by residues of permanent paralysis.

Moreover the monkey at times develops milder forms of the infection in which the paralysis is wanting or is of limited extent, in which cases recovery is the rule. In these latter instances a few days may witness the onset, extension, and disappearance of the paralysis. This class of infections has been compared with the abortive cases of the disease in man. Both in man and the monkey humoral antibodies tend to accompany these milder, as they do the more severe, forms of infection.

The recognized portal of entry of the virus in man is the olfactory nasal membrane, and the instillation of virus into the nares of monkeys is an effective way of producing infection. In the study of reinfection in monkeys which follows, the virus has been inoculated intranasally. This form of instillation avoids all injury of tissue and by employing the olfactory nervous tract for the introduction of the virus into the brain and spinal cord, places it in that peculiar relation to the ordinary defensive mechanisms of the body which we assume to occur in the course of the human infection.

The inoculation of monkeys is made with quantities of virus which are much greater than operate to produce infection in man. The limits of activity of the virus instilled nasally are wide, but they are less wide than with other neurotropic viruses. There is no appreciable difference in the infective effects between a virus containing 1 and 10 per cent of the spinal cord of previously infected monkeys, and there is little difference between the effects produced by one or two and six or seven daily instillations of the virus suspension. But the virus itself displays puzzling and capricious fluctuations of activity which make consistent experiment difficult. The nature of the causes which are responsible for the changes in activity is not known. The several kinds of virus employed in the experiments reported were obtained

from human cases of poliomyelitis occurring in 1909 and the succeeding epidemic years to about 1920, and later in 1928, 1931, 1933, 1934, and 1935.

The specimen now called mixed virus was first successfully passaged in monkeys in 1909, and as its activity fluctuated, strengthened by additions of active specimens as they were secured up to 1920 or thereabouts. Mixed virus is therefore a polyvalent specimen, a fact which is not to be lost sight of in considering the protocols to follow. The mixed virus contains no admixture of specimens secured in the epidemic years in New York of 1928, 1931, 1933, and 1935, the epidemic year in Philadelphia of 1932, and the Cuban epidemic year of 1934. Although it will appear that certain of the recovered monkeys were originally inoculated with specimens of 1928, 1931, 1933, and 1935, it will also appear that the specimens of these dates were rarely used for reinoculation for the reason that they have fallen below the standard of activity or virulence demanded for these tests.

The results of the nasal instillations of the virus into monkeys differ in an essential way from the results of the chance effective entrance of the virus into the nose in man. The incidence of frank poliomyelitis in children even in epidemic years is low; the production of frank symptoms by the inoculations in monkeys is high—up to 80 to 90 per cent of the monkeys instilled. While this difference is a great advantage to the experimenter, it is desirable to keep in mind that there is something essentially artificial in the experiments on reinfection which are to be presented. Despite these considerations we are of the belief that the experiments have an important bearing on present day discussions of immunity in poliomyelitis.

The forms of the disease as observed in man and produced experimentally in monkeys are brought into closer harmony by the studies made on the cerebrospinal fluid of inoculated animals. Changes commonly occur in the fluid consisting first of mononuclear cell increases and second, and less frequently, of the appearance of globulin. The increase in cells arises quickly and tends to precede the onset of clinical symptoms and may occur without any other sign of infection being detected. Interesting as is this phenomenon in normal or previously uninoculated monkeys (4), it is even more informing as it occurs in animals which have already passed through one or even two attacks of paralytic poliomyelitis.

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The employment of recovered or convalescent monkeys for reinoculation has been turned to another account, namely, the consideration of the occurrence of distinct immunologic strains of virus. The protocols which follow are arranged so as to show whether reinfection was induced by a corresponding (homologous) or differing (heterologous) virus.

EXPERIMENTAL

Primary Attack.—It is immaterial to the problem of reinfection how the first, or primary, attack of poliomyelitis was induced. The virus is capable of using any nerve route for ascent to the central nervous organisms (5). For the most part the original attack followed from intracerebral inoculation, but it also followed nasal instillation, subcutaneous or even intravenous injection of the virus.

Clinical Records.—The effects of the instillations were determined by daily examinations consisting of direct observation, rectal temperature readings taken at the same hour each day, and systematic examination of the cerebrospinal fluid withdrawn by cisternal puncture. The inoculated monkeys were released one at a time into a wire enclosure large enough for the attendant to enter. Any awkwardness in running and climbing could be quickly determined by the trained observer and the voice, state of the hair, and other peculiarities accurately noted. Prior to the virus instillations the cells in the cerebrospinal fluid were counted once or oftener and the countings were repeated at 48 hour intervals after the inoculations. Experience had taught us that cisternal punctures alone and the nasal instillation of inactive substances, such as physiological salt solution or suspension of virus of low virulence, do not affect materially the cell count. For the detection of globulin, Noguchi's butyric acid reagent was used.

Virus Suspensions.—The suspensions, varying in concentration from 1 to 10 per cent, were prepared from glycerolated monkey spinal cord and medulla previously washed in two changes of salt solution. The crude suspensions were centrifuged lightly (300 revolutions per minute) for 1 minute, and a new suspension was made for each instillation. The glycerolated specimens were less than 10 weeks old. The non-etherized monkey is held in the upright position with head bent backward. An ordinary rubber urethral tip is attached to the dropper and placed tightly against the nostril, when 1 cc. of the suspension is forced into the nose. The fluid distributes itself over the nasal membrane, the excess, which is small, escaping into the nasopharynx. The indications are that the swallowed or even aspirated suspension is without pathogenic effect.

Cisternal Puncture.—The monkey is thoroughly etherized and the back of the neck shaved and cleaned (surgical sterilization). An assistant places it on its abdomen with the head dropping over the edge of the table, the head being firmly held with both hands. The hypodermic needle (1 inch cannula, No. 20 gauge) is inserted vertically almost its entire length until it reaches the cisterna

TABLE I
Second Attacks, Homologous Reinfections

Experiment	1st attack	2nd attack	Neutralization	Comment
I 11/9/12	Intracerebral injection of M.A. virus. Arms and legs paralyzed. Recovery complete	11 mos. rest period. Reinforcement subcutaneous injections of M.A. virus, 440 cc. 5% suspension, from 7/23 to 11/11/13. Sudden development of paralysis on 11/15, and death	None	The dose of virus was increased in last 4 wks. followed by the reinfection
II 10/13/13	Intracerebral injection of M.A. virus. Partial paralysis of arms and legs and facial paralysis. Recovery with residue	Subcutaneous injection of M.A. virus, 5 cc. 5% suspension, 12/16 and 12/19/21. Paralytic symptoms and death	"	Possibly an instance of induced relapse rather than of 2nd attack*
III 12/23/33 <i>Macacus cynomolgus</i>	Intracerebral injection of mixed virus, 12/31/33 to 1/2/34. Progressive symptoms. Recovery complete	2 nasal doses of mixed virus, 2/19 and 2/21/34. 2/24 beginning symptoms; increase, prostration, and death. Cells rose from 34 and 30 to 320	"	Typical reinfection
IV 12/27/33 to 1/2/34	5 nasal doses of 1933 virus. Cells rose to 335. No clinical symptoms	6 nasal doses of 1933 virus, 2/5 to 2/10/34. Cells rose from 67 to 620. High cell count and fever (105°) coincided. Paralysis and death	"	Example of symptomless involvement of cerebrospinal fluid followed by typical attack

* Cases of relapse in man and monkey are collected in Poliomyelitis, International Committee for the Study of Infantile Paralysis, Baltimore, 1932, 93 and 187. Lovett's case cited as an instance of second attack by Amoss, in Rivers' Filterable viruses, Baltimore, 1928, 179, is probably another instance of relapse. The quiet period between attacks of 2 years adopted by Still for distinguishing between relapses and reinfections is a useful arbitrary device.

TABLE II
Second Attacks, *Heterologous Reinfections*

Experiment	1st attack	2nd attack	Neutralization	Comment
V 11/6/33	Intracerebral injection of 1933 virus, 11/6 and 11/15 (accelerating dose). Progressive symptoms with deltoid paralysis. Recovery	2 nasal doses of Philadelphia virus, 3/7 and 3/9/35. Cells rose from 21 to 760. Globulin +. Paralysis and death	None	Typical reinfection
VI 12/1/33	Intracerebral injection of 1933 virus with acceleration. Excitement, tremor, ruffled hair. No paralysis	2 nasal doses of Philadelphia virus, 1/24 and 1/26/35. Cells rose to 320. Paralysis and death	After recovery from 1st attack, Philadelphia virus not neutralized	2nd, paralytic, attack following abortive attack
VII 12/23/33	Intracerebral injection of mixed virus. Excitement and tremor. No frank paralysis	2 nasal doses of Philadelphia virus, 1/24 and 1/26/35. Cells rose from 30 to 580. Paralysis and death	None	"
VIII 12/26/33	2 nasal doses of 1933 virus. Excitement, tremor, double ptosis. Cells rose from 48 to 640. Recovery. 2 subsequent nasal instillations: (a) 1933 virus (homologous); cells rose from 82 to 105; (b) Philadelphia virus; cells rose from 26 to 64	3 nasal doses of Havana virus, 5/15 to 5/21/35. Cells rose from 33 to 580. Globulin +. Paralysis and death	3 separate bleedings. Philadelphia virus neutralized; mixed virus not neutralized	2nd paralytic attack following previous mild paralytic one
IX 12/26/33	2 sets of nasal doses of 1933 virus, 12/33 and 2/34. Cells in first series rose from 44 to 465; in second series from 50 to 125. No clinical symptoms	3 nasal doses of Havana virus, 5/15 to 5/17/35. Cells rose from 27 to 670. Paralysis and death	None	Symptomless changes in cerebrospinal fluid followed by typical paralytic attack. Havana virus highly virulent specimen

X 3/9/34	Intravenous inoculation of 1933 virus after normal horse serum intraspinally. Paralysis, prostration, recovery	2 nasal doses of Philadelphia virus, 6/24 and 6/26/35. Cells rose to 690. Temperature 106.6°. No paralysis	Serum taken after 1st attack did not neutralize Philadelphia virus	2nd attack abortive
XI 6/26/34	Intracerebral injection of 1931 virus, 6/26/34; acceleration, 7/3. Paralysis and recovery. 3 nasal doses of Philadelphia virus, 3/7 to 3/13/35. No cell rise or clinical symptoms. 4 nasal doses of Havana virus, 5/15 to 5/24/35. No cell rise or clinical symptoms	2 nasal doses of mixed virus, 11/6 and 11/8/35. Cells rose from 16 to 930. Paralysis and death	Serum taken after recovery from 1st attack neutralized Philadelphia, and did not neutralize mixed virus	Typical reinfection
XII 10/10/34	Intracerebral injection of 1928 virus. Paralysis with recovery. 2 nasal tests: (a) Philadelphia virus, 3/7 to 3/9/35; no effects; and (b) Havana virus, 5/15 to 5/24/35; temperature 105°. No clinical symptoms	2 nasal doses of mixed virus, 11/6 and 11/8/35. Cells rose from 32 to 834 with coincident temperature of 106.8° followed by paralysis and death	Serum taken after 1st attack did not neutralize Philadelphia virus; and serum taken after first 2 unsuccessful installations did not neutralize mixed virus	"
XIII 11/13/35	Intracerebral injection of 1935 virus with acceleration. Paralysis with recovery	2 nasal doses of mixed virus, 3/17 and 3/19/36. Cells rose from 9 to 540. Paralysis followed by recovery. 2 nasal doses of Philadelphia virus, 6/17 and 6/19/36. No cell rise or symptoms. 6 nasal doses of mixed virus, 12/1 to 12/3/36. Cells rose from 22 to 104. 2 accelerating doses, 12/10 and 12/11/36, during fall in cells. No effect and no other clinical signs. 6 nasal doses of Philadelphia virus, 1/13 to 1/15/37. Cells rose from 24 to 360. No fever	Serum taken after 1st attack did not, after 2nd attack did neutralize mixed virus. 2nd serum neutralized Philadelphia virus	"

TABLE II—*Concluded*

Experiment	1st attack	2nd attack	Neutralization	Comment
XIV 4/6/36	Intracerebral injection of mixed virus. Paralysis and recovery	2 nasal doses of Philadelphia virus, 6/17/36. Cells rose to 660. Paralysis and recovery. 4 nasal doses of mixed virus, 11/4 to 11/5/36. Cells rose to 135. No clinical symptoms. 4 nasal doses Philadelphia virus, 12/8 to 12/9/36; accelerating dose, 12/19/36. Cells rose to 245 and temperature to 105°. No other symptoms	Serum taken after 1st attack did not neutralize Philadelphia virus; serum taken after 2nd attack did not neutralize mixed virus	Typical reinfection
XV 10/20/33	Intradermal injections of 32 cc. human 1933 virus, 10/20, 12/13/33. 6 nasal doses of 1933 passage virus, 1/31 to 2/5/34. Cells rose to 1150. Globulin +. Paralysis. Recovery with residues	3 nasal doses of mixed virus, 1/8 to 1/12/35. Cells rose to 880. Globulin +. Paralysis followed by recovery. Second set of residues. Nasal instillations as follows: Philadelphia, 6/24 to 6/29/35, no cell rise; Philadelphia, 12/9/35, cells rose to 127; Havana, 1/14/36, cells rose to 145; no other symptoms in any test; intracerebral injection of mixed virus, 6/2/36, no symptoms. 6 nasal doses of mixed virus, 12/17 to 12/19/36. Cells rose from 18 to 55. No clinical symptoms. 6 nasal doses of Philadelphia virus, 1/26 to 1/28/37. Cells rose from 28 to 102. No fever	After 1933 intradermal injections serum did not neutralize mixed virus. After recovery from 2nd (mixed virus) paralysis, serum neutralized mixed virus	"

TABLE III
Reinforced Monkeys Responding to Reinoculation. Heterologous

Experiment	1st attack	2nd attack	Neutralization	Comment
XVI 3/5/35	Intracerebral injection of Havana virus. Abortive attack. Temperature 106°. Recovery. Subcutaneous injections of 50 cc. 5% suspension Havana virus, 4/8 to 5/10/35	2 nasal doses of Philadelphia virus, 6/24 and 6/26/35. Cells rose from 28 to 240. Paralysis and death	None	Typical reinfection
XVII 3/5/35	Intracerebral injection of Havana virus. Abortive attack. Recovery. Subcutaneous injections of 50 cc. 5% suspension Havana virus, 4/8 to 5/10/35. 6 nasal doses of Havana virus, 1/14 to 1/25/36. Cells rose from 30 to 835. No fever or other symptoms. Cells 74 to 83% polymorphonuclears. No bacteria	2 nasal doses of mixed virus, 3/17 and 3/19/36. Mononuclear cells rose from 35 to 785. Arm and leg paralysis. Recovery. Later died of tuberculosis	"	"
XVIII 3/4/31	Subcutaneous injection of aluminum hydroxide and mixed virus, 4/20 to 4/26/32. 7 nasal doses of mixed virus. Paralysis. Recovery with residues. Subcutaneous injections of 11/20 cc. 5% suspension mixed virus, 10/2/31 to 2/14/33. No symptoms	4 nasal doses of Philadelphia virus, 3/27 to 4/10/33. Paralysis with recovery. 4 nasal doses of 1933 virus, 1/10 to 1/13/34. Cells rose to 460 and temperature to 104.2°. Later died of tuberculosis	Serum after reinforcement neutralized mixed virus; did not neutralize Philadelphia virus. Serum taken after 2nd attack did neutralize Philadelphia virus	Typical reinfection in hyperimmune monkey

TABLE IV
Reinforced Monkeys Resisting Reinoculation. Homologous

Experiment	1st attack	Nasal instillation	Neutralization	Comment
XIX 6/30/31	Cisternal injection of mixed virus. Paralysis with recovery. Subcutaneous injections of 1120 cc. 5% suspension mixed virus, 10/2/31 to 2/11/33. No symptoms	4 nasal doses of mixed virus, 3/27 to 4/10/33. No cell count. No clinical symptoms. Test not repeated	Reinforced serum neutralized mixed virus	The failure to count cells and repeat the test with other specimens of virus leaves experiment inconclusive
XX 5/7/31	Cisternal injection of mixed virus. Paralysis and recovery. Subcutaneous injections of 970 cc. 5% suspension mixed virus, 10/2/31 to 12/2/32. No symptoms	6 nasal doses of mixed virus, 12/27/32 to 1/3/33. No cell count. No symptoms. Test not repeated	" "	" "
XXI 12/8/32	Intracerebral injection of Philadelphia virus. Paralysis with recovery. Reinforced with 255 cc. 5% suspension of Philadelphia virus	4 nasal doses of Philadelphia virus, 3/27 to 4/10/33. No cell count. No symptoms. Test not repeated	Reinforced serum neutralized Philadelphia virus	" "

TABLE V
Reinforced Monkeys Resisting Reinoculation. Heterologous

Experiment	1st attack	Nasal inoculation	Neutralization	Comment
XXII 2/9/31	Intracerebral injection of mixed virus. Marked non-paralytic infection. Recovery. Reinforced with 1120 cc. 5% suspension mixed virus. Companion to Experiment XVII	4 nasal doses of Philadelphia virus, 3/27 to 4/10/33. No cell counts. No symptoms. Test not repeated	Serum taken prior to nasal test neutralized Philadelphia virus	The failure to count cells and to repeat nasal test leaves experiment inconclusive
XXIII and XXIV 1/7/32	Intracerebral injections of 1931 virus. Both monkeys developed paralysis and both recovered. Reinforced with 180 cc. 5% suspension 1931 virus given subcutaneously	4 nasal doses of Philadelphia virus, 3/27 to 4/10/33. No cell counts. No symptoms. Test not repeated	Serum taken before reinforcement did not neutralize mixed virus; serum taken after reinforcement neutralized Philadelphia virus	"
XXV 10/7/32	7 nasal doses of Philadelphia virus. Paralysis and recovery. Reinforced with 255 cc. 5% suspension Philadelphia virus given subcutaneously	4 nasal doses of mixed virus, 3/27 to 4/10/33. No cell counts. No symptoms. Test not repeated	Reinforced serum neutralized Philadelphia virus	"

TABLE VI
Originally Resistant Monkeys

Experiment	Preparation	Testing	Neutralization	Comment
XXVI and XXVII 5/20/32	2 <i>Macacus rhesus</i> of same weight given subcutaneously 1250 gm. of dry acetone precipitate mixed virus	<p>Monkey XXVII given 6 nasal doses of mixed virus, 12/27/32 to 1/3/33. Paralytic poliomyelitis, death. Monkey XXVI inoculated as follows: (a) Nasal doses of mixed virus, 3/27 to 3/28/33; accelerated, 4/8 to 4/10. No effect. (b) 4 nasal doses of mixed virus, 1/10 to 1/13/34. Cells rose to 285. No clinical symptoms. (c) 4 nasal doses of mixed 1928 and 1933 virus, 1/29/34. Cells rose to 125. Evanescent weak arms. Accelerating nasal doses, 2/2 and 2/3. Cells rose to 385. No clinical symptoms</p>	<p>None</p> <p>Serum taken on 4 occasions from monkey XXVI failed to neutralize mixed virus</p>	<p>In all series of experiments with <i>Macacus</i> monkeys occasionally highly resistant animals are encountered. The acetone precipitated virus produced no immunity. One monkey was of average, the other of greater natural resistance. The sole evidence of latter's response to the inoculations was revealed by the cell counts</p>

magna region. Sudden release of pressure indicates the penetration; the stylet is removed and the fluid allowed to escape, collected by means of a capillary pipette, and placed in small Wassermann tubes. When blood-tinged fluid appears, pipetting is carried on until the specimen becomes clear before the collection.

DISCUSSION

The tabulations constitute the experimental data on the reinfections. The abbreviated protocols leave no doubt that second attacks of poliomyelitis can be induced in monkeys by using the nasal mode of inoculation. And the occurrence is not exceptional, although it is also not invariable. The proportion of convalescent monkeys which responds under favorable conditions of experiment is high. Certain convalescent monkeys respond to the first instillations, others only after successive instillations. We have already recorded (4) that monkeys not previously instilled will resist one and respond to another instillation sometimes, so that it is not surprising that the identical set of reactions should occur in the convalescent monkeys.

Fortunately, the tests were numerous enough to cover the experimental disease in its main manifestations: mild, almost undetectable cases; typically abortive cases; and cases as severe as are ever encountered. It cannot, therefore, be argued that only the light convalescents are subject to reinfection.

It must, however, be kept in mind that the test used is a severe one. The amount of virus instilled is huge compared to anything likely to happen in man. On the other hand, the monkey, compared to man, is relatively an insusceptible species. Contact, cage infections in monkeys practically do not occur. The very rare such instances which have been reported among the thousands of inoculated monkeys serve merely to emphasize the strong natural resistance they display. On the other hand, the disease in monkeys is severer, as a rule, than the human malady. The defensive mechanism, once broken down in the monkey, is less competent than that of man.

Compared to second infections so far recognized in man, the tests with monkeys would suggest that they are the more susceptible species. No one knows, of course, what might happen in man if convalescents received such vast doses of virus intranasally. The two sets of conditions cannot, therefore, be compared numerically. What the experiments in monkeys can be made to do is to arouse greater

interest in cases of second attack in man and possibly reveal the mechanism involved.

The cases of second attack in man may safely be assumed to express themselves in other ways than in frank paralysis as they do in monkeys. All the examples of reinfection in man so far reported are of the paralytic variety. The study of the reinoculated convalescent monkeys has shown conclusively that milder effects arise which are detectable only through the changes occurring in the cerebrospinal fluid accompanied by fever temperatures. It is a curious fact that the cerebrospinal fluid which never harbors the virus should register so readily the presence of virus on the nasal membrane. Incidentally, the fact should be emphasized that the height of this cell reaction in the fluid often coincides with fever temperatures either preceding the onset of obvious clinical symptoms or occurring in the absence of those symptoms.

That the presence of humoral antibodies is an insufficient bar to the penetration of virus from the nose to the brain, many recent studies on actively immunized monkeys have shown (6). This finding, highly important in relation to efforts made to produce effective vaccines, is confirmed in an interesting manner by the further finding that convalescent monkeys in which the titre of humoral antibodies has been increased by reinforcement or hyperimmunization respond to nasal instillations with typical paralytic second attacks. The tables contain brief protocols of two kinds of reinforced monkeys—those which did and those which did not respond with reinfection symptoms. It will be noted that the failures to respond consist of animals nasally instilled only once and in which the cerebrospinal fluid was not examined. The reason for this disparity is that these monkeys were among the early convalescents studied and before the technical methods later employed had been put into use.

There is probably nothing strictly accidental in the failure of nasal instillation at one time and its effectiveness at another. One specimen or strain of virus may well be better suited to induce infection in a given convalescent animal than is another, but the degree of virulence irrespective of specimen or strain undoubtedly plays an essential part in the result. Note should be taken of the fact that in the long run only two specimens of virus proved strictly reliable—mixed virus and

Philadelphia virus, to which for a short time the Havana strain could be added. But not all passages of any of these viruses were uniformly effective. Fluctuations already referred to still occurred to disturb the expected results. The 1933 virus was highly virulent for a time, only to fluctuate widely and to sink to so low a level that it was never possible to re-establish it.

Can no more than two attacks of paralytic poliomyelitis be induced in monkeys? Still (3) in his collected cases includes one of a third attack in a child. It is still too early to make confident statements about third or further attacks in the monkey. So far we have had available three monkeys which, having passed successfully through two paralytic attacks, have been subjected to additional inoculations. None developed a third paralytic attack, but all responded with increases in cells in the cerebrospinal fluid. The monkey in Experiment XIII, after a rest period of $6\frac{1}{2}$ months, reacted to mixed virus; the monkey in Experiment XIV, after a similar rest of $4\frac{1}{2}$ months, reacted successively to mixed and Philadelphia virus; and the monkey in Experiment XV, after a rest of $6\frac{1}{2}$ months, reacted mildly to mixed virus. In these three instances the virus specimens used corresponded with those which had previously produced paralysis. After a further rest interval the three monkeys will be instilled again with a heterogeneous specimen. Knowledge is still too small to permit of generalizations concerning an induced nervous tissue immunity in opposition or in addition to a humoral immunity (7). We do know that the virus has a wide distribution in these tissues even in monkeys with localized paralysis. Certain animals possess naturally high nervous tissue immunity. One such instance has been recorded (Experiment XXVII). The problem of local immunity of the nervous tissues, natural or induced, is one to be left to future study.

So long as knowledge of the variety of the virus is so small, we should speak of particular or specific strains with caution. There are reasons to believe that passage viruses differ somewhat from each other immunologically and that a given specimen of human virus changes in the course of monkey passages (8). To designate the specimens derived from epidemics in different years or places as strains is to employ a rough classification only. In the experiments reported, the mixed virus and the Philadelphia virus have exhibited contrasting powers of

infection to which, for convenience, the terms homologous and heterologous have been tentatively applied. When we pass from the infective properties of these specimens to the more subtle immunological properties the difference still holds in spite of occasional overlapping as revealed by the neutralization test.

Finally, the protocols show that reinfection is capable of being induced by the same (homologous) specimen with which the original infection was produced, as well as with alien (heterologous) specimens of different origin. The position to be assigned the Havana virus is still undetermined. Its effectiveness in the few instances in which other virus specimens proved powerless may be due more to the degree of virulence than to true heterology.

CONCLUSIONS

Monkeys which have recovered from an attack of experimental poliomyelitis are subject to reinfection by the nasal route.

Second attacks of the disease result from inoculation with the specimen of virus used to produce the first attack and with specimens of different origin.

Reinfection takes place in monkeys which have recovered from mild and from severe attacks and in convalescent animals which have been subjected to hyperimmunization.

The 2 year quiet period proposed by Still to separate relapses from second attacks, judging from the monkey, is probably excessive. Until greater attention is given the reinfections of varying intensities in man, conclusions on this point must be wholly tentative.

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THE CULTURE OF WHOLE ORGANS

I. TECHNIQUE OF THE CULTURE OF THE THYROID GLAND

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PLATES 17 TO 19

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The culture of whole organs (1) in the Lindbergh apparatus (2) has required the development of techniques that vary in some manner according to each organ. The purpose of this article is to describe the procedures used in the perfusion of the thyroid gland, the accidents that may happen during the operation, and the effects of the perfusion upon the gland.

Technical

The technique consists of several parts: preparation of the gaseous medium, preparation of the fluid medium, preparation of the Lindbergh perfusion pump, transplantation of the gland into the organ chamber, perfusion, and removal of the gland from the pump.

1. *Preparation of the Gaseous Medium.*—The gaseous medium is composed of nitrogen, oxygen, and carbon dioxide in various proportions. Generally, a mixture consisting of 80 per cent oxygen, 3 or 4 per cent carbon dioxide, and 16 or 17 per cent nitrogen is used. The mixture is made by introducing into a steel gas cylinder equipped with a pressure gauge the three gases under partial pressures of 80, 3, and 17 pounds, respectively. Thus, the cylinder contains the proper gas mixture under a pressure of 100 pounds. After a period of 24 hours, during which the gases are allowed to become thoroughly mixed, a gas pressure reducing valve is attached to the cylinder. When necessary, the composition of the mixture is ascertained by analysis. Before the experiment is started, the gas is led into the incubator and to the inner chamber of the oil flask (2).¹

2. *Preparation of the Fluid Medium.*—The volume of the medium is approximately 200 cc. for a gland weighing 100 mg. It varies generally from 225 cc.

¹ Lindbergh (2), p. 425.

to 250 cc. The medium consists of blood serum more or less diluted with Tyrode solution, or of artificial nutrient solutions containing only a small amount of blood serum. Phenol red is added at a concentration of 0.005 gm. per 100 cc. The fluid is sterilized by filtration. The filtering apparatus consists of an Erlenmeyer flask to which have been attached a lateral outlet for applying vacuum, and a curved fitting near the bottom of the flask for evacuating the fluid. Into the neck of the flask is introduced a Berkefeld filter mounted on a rubber stopper. The lateral outlet bears a rubber tube and a glass bulb filled with non-absorbent cotton. The lower outlet is connected by a short piece of rubber tubing, compressed by a clamp, with a cannula protected by a glass bell. The apparatus is prepared for sterilization by completely covering the bell with non-absorbent cotton enveloped in gauze. Then it is wrapped in paper and sterilized twice for 1 hour in an autoclave under a pressure of 17 to 18 pounds.

Before filtration, the pH of the fluid is brought to about 6.9 or 7.00 by bubbling through it a mixture of 92 per cent oxygen and 8 per cent carbon dioxide. The filtering apparatus is mounted on an appropriate stand. A funnel is connected by a short rubber tube with the Berkefeld filter. The lateral outlet is attached to the vacuum line through the bulb filter. The fluid is placed in the funnel and filtered into the flask. After filtration, the pH of the fluid is about 7.3 or 7.4. Careful examination shows whether there are any foreign particles floating in the field.

3. Preparation of the Lindbergh Perfusion Pump, and Introduction of the Fluid Medium into the Lower Reservoir.—The perfusion pump has been washed with chromic and sulfuric acid for 2 hours, and rinsed with running filtered water for 6 hours. Then it is dried by vacuum, the air being aspirated after double filtration, first, through a cotton bulb filter and second, through a Berkefeld filter. These operations should be carried out as described by Lindbergh (2).² It is indispensable for the surface of the glass to be strictly clean, lest particles of dust form emboli in the small arteries and capillaries of the gland. Once thoroughly dried, the pump is prepared for sterilization by protecting all the openings with non-absorbent cotton and gauze plugs and pads. Then it is wrapped in paper. The rubber stoppers containing the glass tubes connected with the bulb filters (2)³ are prepared separately for sterilization in Petri dishes covered with gauze and cotton pads. The pump is placed in an autoclave, which is brought to a temperature of 160°C. by introducing steam at a pressure of 75 pounds into the outer jacket. No steam is present in the sterilizing chamber. In case an autoclave of this type is not available, an electric oven can be used as a substitute. The sterilization lasts for 4 hours.

After the pump has cooled, the fluid medium is introduced into the reservoir chamber through the neck of the equalization chamber (2).⁴ The filtering appa-

² Lindbergh (2), p. 426, Operating Directions.

³ Lindbergh (2), p. 415, Chart 2, Nos. 1, 2, 12, 22.

⁴ Lindbergh (2), p. 415, Chart 2, No. 11.

ratus is fixed to a stand in such a manner that the cannula and its bell are at the level of the neck of the equalization chamber of the pump. The gauze and cotton pads covering the neck of the pump and the bell of the filtering apparatus are removed simultaneously, and the cannula is introduced into the neck of the apparatus. This procedure renders infection practically impossible. Then the clamp is released and the fluid flows through the equalization chamber into the reservoir chamber. As the lateral outlet of the Erlenmeyer flask is protected by the bulb filter, there is no danger of contamination of the surface of the fluid by the air of the room. The necks of the equalization and reservoir chambers are now closed with rubber stoppers equipped with bulb filters, and sealed with waterproof cement. The opening of the organ chamber is left protected by cotton and gauze plugs and pads. When the fluid is subjected to quantitative analysis, it must be accurately measured. It is caused to flow from the filtering apparatus into a graduated tube. A funnel, protected by cellophane, is placed in the neck of the reservoir chamber (2),⁵ and the fluid is poured into it, also under protection of cellophane.

4. *Preparation of the Thyroid.*—Cats and rabbits are generally used, less frequently, fowls. The technique is similar to that developed long ago for the transplantation in mass of organs (3). That is, the gland is removed, together with a segment of carotid artery in cats and rabbits, and of aorta in fowls, and with the surrounding connective tissue. The operative procedures require a higher degree of asepsis than an ordinary surgical operation, because an organ does not protect itself against infection in the perfusion pump as it does in the body. When the air of the room is not free from bacteria, antisepsis, as well as asepsis, must be used in the surgical procedures. During the handling of the blood vessels, special care is taken to prevent emboli due to the coagulation of the blood, to foreign bodies, or to air. As the thyroid artery of cats and rabbits is very small, it must be left undisturbed in the connective tissue and protected from antiseptics used during the operation. In sum, every detail of the technique must conform to the well known principles of vascular surgery.

(a) The skin of the neck of an etherized cat or rabbit is shaved, and sterilized with Dakin solution. Chloramine can be substituted for Dakin solution. Tincture of iodine is not used, because a large amount of iodine may go through the skin into the blood. The animal is bled to death from the femoral artery. The operating field is circumscribed with sterile black towels.

(b) At the time of death, or immediately after, a longitudinal incision from the chin to the sternum is made. The skin is dissected on each side and cut transversely in order that the flap can be everted. The trachea and muscles, and the edges of the operating field are scrupulously protected by pads squeezed out of antiseptic solution. In fowls, the left side of the thorax is opened along its whole length by an incision of the skin and muscles and section of the ribs.

(c) Incision of the muscles and exposure of the carotid artery and vagus nerve.

⁵ Lindbergh (2), p. 415, Chart 2, No. 18.

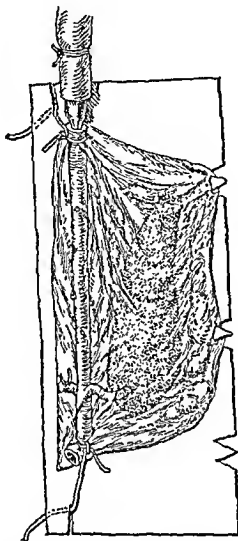
The artery and the nerve are covered by a pad wet with Dakin solution. The aponeurosis is cut on the external side of the vagus nerve, which is not dissected from the artery. The vagus nerve is severed in the lower part of the neck and separated from the artery on a length of 1 cm. A silk thread that has been soaked in Dakin solution is placed under the artery. This thread will later be used to tie the cannula in the artery. When a fowl is used, the heart, the aorta and its branches, and the thyroid are exposed by stretching the thoracic opening with a Gosset retractor. A silk thread is placed under the aorta.

(d) After ligation of the posterior collateral, which branches from the carotid at the level of the thyroid artery, the upper part of the vagus is severed. At a point located about 3 cm. above the thyroid artery, a thread wet with Dakin solution is placed under the carotid and left provisionally untied. The connective tissue between the carotid artery, the vagus nerve, and the thyroid gland is undisturbed.

(e) Introduction of the cannula into the artery and perfusion of the gland. Glass cannulas of appropriate size, mounted on a piece of pure gum tubing, the total length of tubing and cannula being 5.5 cm., have been sterilized in a small, round dish in Ringer solution. They lie full of fluid on the bottom of the dish. The solution has been filtered with extreme care, and is absolutely free from any floating particles. A Gentile syringe, the bulb separated from the glass chamber, has been sterilized in another jar. It is removed from the jar under the protection of a veil of sterile cellophane and filled with Ringer solution. This Ringer solution has been filtered through a Berkefeld filter and through a quartz sand filter, and must be completely free from floating particles. If any blood has remained in the carotid, it is pushed by pressure of the index finger toward the distal end of the artery. A small transverse incision is made with the scissors in the proximal part of the carotid. A cannula is taken with a mosquito forceps from the jar, the rubber tubing being clamped while the cannula is still under the fluid. In this manner, no air penetrates either into the cannula or into the rubber tubing, and the possibility of air embolus is removed. The cannula, protected against atmospheric dust by a gauze pad soaked in Dakin solution, is introduced into the artery, and fixed by tying the silk thread. One end of the thread is cut close to the knot. The other, at a distance of about 3 cm. The same procedure is applied to the introduction of a cannula into the aorta of a fowl.

(f) Washing the blood out of the artery. The tip of the Gentile syringe is introduced into the rubber tubing of the cannula. The bulb is gently compressed in order to push the blood contained in the carotid artery and the small air bubbles that may have remained in the cannula into the distal part of the carotid, beyond the location of the future ligature. Then the artery is inspected along its entire length, in order to make sure that no foreign bodies, blood clots, or air bubbles are present. Afterwards, the thread lying on the distal part of the carotid artery is tied. By exercising more pressure on the bulb of the Gentile syringe, the gland is perfused with Ringer solution for a few seconds in such a

manner as to expel all remaining blood. Then the syringe is removed while the bulb is being pressed, in order that the cannula and the rubber tubing may remain full of fluid. Care is taken afterwards not to make pressure on the rubber tubing in order to keep it full of fluid. In fowls, the blood of the aorta is



TEXT-FIG. 1. Semischematic drawing. Mica holder with its upper and lower slits, and its three saw teeth. Carotid artery fixed on the holder by threads inserted in the slits. Gland suspended by attaching the surrounding connective tissue to saw teeth.

washed in the same manner. Then the distal ends of the arteries are ligated at a short distance from the edges of the thyroid.

(g) Section of the carotid and removal of the gland. The sternohyoid muscle is severed at the lower part of the neck, taken between the jaws of an artery forceps, and dissected out from the trachea and from the anterior surface of the thyroid gland to its upper insertion. Then it is cut and removed. The distal and proximal ends of the carotid artery are severed. The connective tissue is cut transversally from the carotid to the trachea. While the carotid and the

gland are protected by a pad wet with Dakin solution, they are seized between the thumb and the index finger and pulled gently away from the trachea. The connective tissue is cut with scissors close to the trachea, the esophagus, and the muscles of the larynx. The gland is now removed in mass with the carotid and the intermediary connective tissue. The thyroid gland of the fowl is very easily removed with the large arteries on which it lies.

(h) Stretching the gland on a bolder. The gland holder is made of a rectangular mica plate, 5.5 by 2.4 cm. (Text-fig. 1). Close to one of the long sides of the plate, on each short side, there is a slit about 0.5 cm. long. The other long side of the plate bears three sets of teeth, like saw teeth. The gland, protected by a pad, is stretched on the bolder. The end of the thread that fixes the carotid to the cannula is inserted into the upper slit in the mica plate. The end of the thread of the distal ligature of the carotid is inserted into the lower slit (Text-fig. 1). In this manner, the carotid is maintained in a straight position without being stretched or twisted. Then the gland and its connective tissue are spread on the surface of the mica. Care is taken not to twist the connective tissue around the carotid segment or the thyroid artery. The thyroid artery is inspected from its beginning on the wall of the carotid artery to its division at the upper part of the gland. The outer part of the connective tissue is hung to the teeth of the holder (Text-fig. 1). Then a large sheet of sterile cellophane is spread over the hands of the operator holding the mica plate, and the preparation is transported to the Lindberg perfusion pump. The thyroid gland of the fowl is placed in the organ chamber without a holder being needed.

5. *Setting the Gland in the Organ Chamber.*—This consists of introducing the mica holder into the chamber, and of connecting the gland cannula with the feeding cannula.

(a) Introduction of the gland into the chamber. The operator stands in front of the culture chamber with the thyroid gland covered by the cellophane veil. An assistant simultaneously removes the pad and the plug protecting the opening of the chamber, and covers this opening with the edge of the cellophane veil. Under the protection of the cellophane, the mica plate is introduced into the chamber and allowed to slide down the tube as far as the platinum screen (2).⁶ In this manner, no infection from the air is possible.

(b) An assistant, by blowing through a rubber tube and bulb filter into the reservoir chamber, causes the fluid medium to flow through the feeding cannula into the organ chamber (2).² The fluid must flow in such a manner that no foaming occurs either in the chamber itself, or in the feeding tube and cannula. When a sufficient amount of fluid has been introduced to wet the sand filter and fill the upper floating valve reservoir (2),^{2, 7} the gland is mounted on the feeding cannula in the following manner. The upper part of the rubber tube is seized between the jaws of a curved forceps, and slipped on the feeding cannula.

⁶ Lindberg (2), p. 415, Chart 2, Nos. 4 and 5.

⁷ Lindberg (2), p. 415, Chart 2, Nos. 9 and 14.

As little space as possible is left between the end of the feeding cannula and the beginning of the arterial cannula. Then, while the chamber is protected by the cellophane veil, the glass stopper, with its glass tubing and filter, is inserted into the opening.

6. *Connecting the Pump with the Pulsating Apparatus.*—(a) The three orifices of the pump are sealed carefully with waterproof cement. The apparatus is placed for a few minutes in the air current produced by a ventilator, in order to dry the cement. It is imperative for the sealing of the openings of the pump and of the points of insertion of the glass tubes into the rubber stoppers to be hermetic.

(b) The perfusion pump is placed in the incubator and connected with the pulsating gas pressure apparatus, in the way already described by Lindbergh (2).^{*} The pulse rate is adjusted, and also the maximum and minimum pressures, according to the nature of the experiment. The pulse rate has generally been 80, the maximum pressure 160, and the minimum pressure 110.

7. *Perfusion of the Gland.*—The perfusion of the gland lasts about a week. During this period, the pulsation rate and the maximum and minimum pressures are observed every day and adjusted, if necessary. The modifications in the pH of the fluid are noted by comparison of the color of standard tubes with that of the feeding tube. Care is taken to prevent foaming and accumulation of the fluid in the chamber. There is need of very little adjustment of the apparatus during the period of perfusion. When the pH goes below 7, the experiment is interrupted.

8. *Removal of the Gland from the Chamber.*—The gland is removed from the chamber either to be placed in another apparatus, or for the final results of the experiment to be studied. When an experiment is to be continued, the fresh pump is placed beside that containing the gland. After the cement has been incised with a knife, the rubber stopper is removed and the opening of the organ chamber carefully flamed with a Bunsen burner. Then both pumps are covered with a sheet of sterile cellophane. With a curved forceps, the gland is unbooked from the feeding cannula, removed from the chamber, and placed in the chamber of the second apparatus, as previously described. If the experiment is completed, the mica holder is removed, the gland dissected, weighed, and fixed in Zenker's formalin. The fluid is examined from the point of view of its physical changes, such as its clearness or opacity, and the presence or absence of floating particles. It is then removed from the pump and submitted to chemical analysis, which will be described in a subsequent article.

The extirpation of the thyroids of a cat or a rabbit, and their setting in two pumps takes about 40 minutes. The technique, in spite of its many details, is simple and can be learned easily in about 2 weeks.

^{*} Lindbergh (2), p. 422.

Accidents

The accidents that may happen to the thyroid during the course of the perfusion are infection, hemorrhage, emboli, and twisting or compression of the thyroid artery.

1. Infection generally is expressed by opacity of the fluid and change in its color. Nevertheless, in extremely rare cases there may be no visible modification of the fluid, the infection remaining confined to the gland, which becomes light yellow. The causes of infection have been bacteria or molds. This complication occurred almost exclusively during the first phase of the development of the technique. It disappeared when the pumps were sterilized at a higher temperature and for a longer time, and when the tissues were more adequately protected against contamination by the use during operations of gauze pads, gloves, thread, and instruments that had been antiseptically treated. The technique could be greatly simplified if the experiments were performed in a totally sterile room. However, in an atmosphere that is not free from bacteria, the above described technique has allowed a series of over 100 experiments to be made without an infection. Therefore, septic complications can be considered to be practically eliminated.

2. In order to be dangerous for the organ, a leakage has to be quite large. However, the posterior collateral of the cat's carotid artery, when not ligated, may cause a loss of fluid capable perhaps of bringing about a partial necrosis of the gland. Hemorrhages of smaller size are not dangerous. No ligatures of the collaterals of the thyroid artery, therefore, need to be made. These collaterals bleed into the connective tissue, which becomes more or less edematous. Such edema has no ill effect on the gland. Severe leakage is more apt to occur in the perfusion of the chicken's thyroid than in that of the cat's or rabbit's thyroid.

3. Emboli have more frequently brought about serious accident. They cause total or partial necrosis of the gland. Three types of emboli have been observed: foreign particles, blood clots, and gas bubbles. Sometimes the fluid used in the perfusion of the gland during the operation was found to contain floating particles. These particles may come from the Berkefeld filter, from the wall of the rubber tubing, from dust deposited by air inside of the pump or filtering apparatus, from cotton and gauze pads. All the rubber tubing used in the apparatus has to be of pure gum, in order that no foreign particles be set free into the fluid. The glassware is washed in filtered water, and dried in filtered air. Cellophane is interposed between the mouth of the jars containing Ringer solution and the pads, in order that the fluid may be protected against cotton or gauze filaments of the pads during sterilization. Finally, by filtering the fluid through quartz sand, and by allowing the particles that may come from the Berkefeld filter to collect on the bottom of the filtering flask, this type of embolus has been almost completely eliminated. Emboli may also come from a blood clot formed in the carotid artery if, after this artery is opened, the blood is not immediately washed

out. The clots are generally large, and the necrosis of the gland is complete. This complication has been eliminated by strict adherence to the principles of blood vessel surgery. When the operation is performed on a dying or dead animal, the carotid artery is almost empty. If a small clot has formed in the carotid artery, it is forced by perfusion with the Gentile syringe into the distal part of the artery, beyond the location of the future ligature.

A large air embolus may also be a serious accident. The air can be introduced into the carotid from the organ cannula and its rubber tubing. This complication also takes place when air bubbles are present in the feeding cannula, or the feeding tube of the pump, or when the rubber tubing of the organ cannula is not completely filled with Ringer solution while it is being slipped over the feeding cannula. Simple procedures have been developed to prevent the occurrence of these accidents. By perfusing the carotid artery with the Gentile syringe before tying the ligature of the distal part of the artery, one causes the air bubbles that may remain in the lumen to be forced from the distal into the upper part of the vessel, beyond the distal thread. This thread must be tied only after inspection of the carotid artery has shown that it is free from blood clots or air bubbles. When the perfusion of the gland with the Gentile syringe is completed, the syringe is removed from the cannula in such a manner, as already mentioned, that the gland cannula and its rubber tubing contain no air bubbles. These various procedures have eliminated, in a large measure, the occurrence of partial or complete necrosis of the gland due to emboli.

4. Twisting or compression of the thyroid artery by connective tissue happens to cat and rabbit thyroids, and not to chicken thyroids. After the gland, its surrounding connective tissue, and the corresponding segment of carotid artery have been removed, the gland may become twisted around the carotid or the thyroid artery. This complication is prevented by spreading the preparation on the surface of the mica bolder, taking pains to respect the normal relations of the vagus, the carotid, and the upper and lower poles of the gland. Each part of the preparation is maintained in its place by appropriate tension of the upper and lower arterial threads, and of the connective tissue hung on the mica teeth of the bolder (Text-fig. 1). The thyroid artery must not be stretched, as in such a case the circulation of the gland is hampered. Another complication has been observed from connective tissue strands compressing the thyroid artery. This happens especially when the connective tissue has been made sticky by too persistent application of pads wet with Dakin solution. This arterial compression is prevented by examining the thyroid artery when it is spread on the bolder, and cutting any strand of connective tissue that may compress it.

Effects of Perfusion on the Gland

As soon as the pump is connected with the second portion of the apparatus (2),⁹ and the maximum and minimum pressure are adjusted,

⁹ Lindberg (2), p. 427.

the carotid artery begins to pulsate. Pulsation can also be detected in the thyroid artery. In a few minutes, the gland swells and becomes pink. There are little spurts of fluid from the small collaterals of the thyroid. Generally, these collaterals bleed into the connective tissue, which becomes more or less edematous. At the beginning of perfusion, the pH of the fluid in the feeding tube is about 7.5. It declines progressively in the course of a week to 7.1 or 7.0, and even lower, according to the metabolic activity of the gland and to the composition of the circulating fluid. The color of the gland becomes pinkish-orange, orange, and yellow. It may be light yellow at the end of a week. Its size remains stationary, decreases, or increases, according to the composition of the medium. Often, these changes cannot be ascertained during perfusion, because the connective tissue is infiltrated by the circulating fluid flowing from small vessels.

The main effect of perfusion is to maintain the gland alive. The comparison of a gland cultivated for 6 days with the gland from the opposite side kept as control shows that there is practically no structural difference when the nutrient medium is diluted serum (Figs. 1 to 4). However, the cultivated glands show a tendency to hyperplasia and to vacuolization of the colloid. When the fluid contained some Witte's peptone, considerable increase in the size of the cells was observed (Figs. 5 and 6). The examination at higher power of the epithelium of perfused glands shows its excellent condition (Figs. 7 and 8). Marked proliferation of the epithelial cells lining the follicles was produced by a medium containing peptone (Figs. 9 and 10). Normal looking follicles were found after perfusions lasting 18 and 21 days. It is obvious that, although the circulating fluid contains neither red blood corpuscles nor hemoglobin, the thyroid does not show morphological signs of degeneration, but remains normal in appearance or undergoes enlargement and proliferation of the cells. Further evidence of the condition of the epithelial tissue after perfusion was given by the cultivation in flasks of small fragments of the glands. An exuberant culture of epithelial cells was obtained from a cat's gland through which diluted serum had circulated for 18 days (Fig. 11). In the same manner, although more rapidly, a very active migration of epithelial cells took place from a fragment of chicken gland, perfused for 3 days with a fluid containing Witte's peptone

(Fig. 12). It is certain that thyroid glands perfused in the Lindbergh pump remain alive.

CONCLUSIONS

1. A technique has been developed for the transplantation of the whole thyroid into the Lindbergh pump, and its perfusion, without the occurrence of infection, emboli, and other complications.

2. The gland remains alive during the period of perfusion, which lasted from 3 to 21 days.

3. The technique is simple enough to be used as a routine procedure for physiological or pathological studies of the thyroid gland.

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EXPLANATION OF PLATES

PLATE 17

FIG. 1. Experiment 40. Left thyroid from cat KT kept as control; not cultivated, fixed in formol immediately after removal. $\times 230$.

FIG. 2. Experiment 40. Right thyroid from cat KT, perfused for 6 days with 40 per cent cat serum. Almost identical with the control. $\times 230$.

FIG. 3. Experiment 65. Left thyroid from cat MO kept as control; not cultivated, fixed in formol immediately after removal. $\times 230$.

FIG. 4. Experiment 65. Right thyroid from cat MO, perfused for 6 days with 40 per cent serum. Many vacuoles in colloid. Gland slightly hyperplastic. $\times 230$.

FIG. 5. Experiment 26. Left thyroid from cat KB kept as control; not cultivated, fixed immediately after removal. $\times 230$.

FIG. 6. Experiment 26. Right thyroid from cat KB, after perfusion for 6 days with medium containing Witte's peptone. Epithelial cells much larger than in control. Gland hyperplastic. $\times 230$.

PLATE 18

FIG. 7. Experiment 274 L. Left thyroid from cat ST, perfused for 6 days with 50 per cent serum. The gland appears normal. $\times 480$.

FIG. 8. Experiment 305. Right thyroid from cat VA, perfused for 6 days with 40 per cent serum. Slightly hyperplastic gland. $\times 480$.

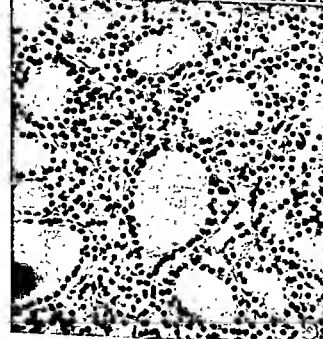
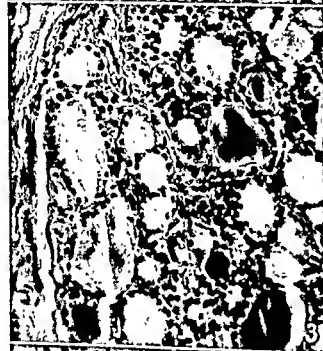
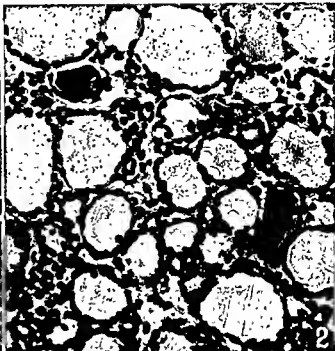
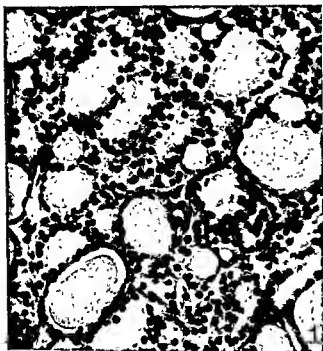
FIG. 9. Experiment 23. Right thyroid from adult chicken kept as control; not perfused, fixed in formol immediately after removal. $\times 230$.

FIG. 10. Experiment 23. Left thyroid from same chicken perfused for 3 days with a medium containing Witte's peptone. Gland shows marked hyperplasia. $\times 230$.

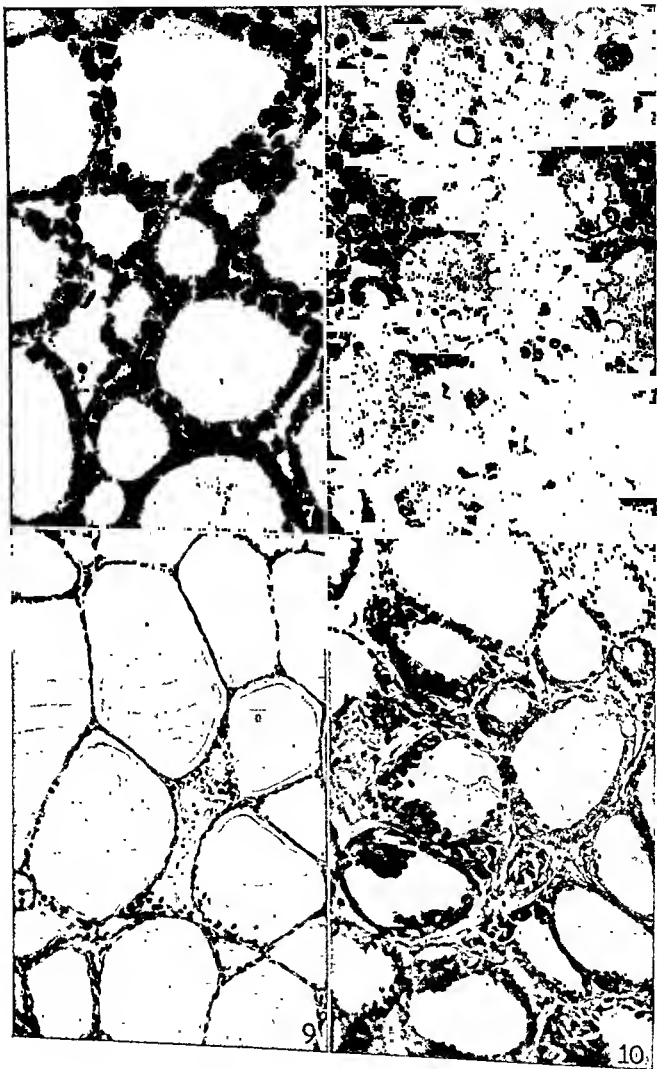
PLATE 19

FIG. 11. Culture in flask of a fragment of a cat's thyroid (Experiment 2), which had been perfused with 40 per cent serum for 18 days. Photograph shows the colony of epithelial cells developed after 6 days' cultivation. $\times 115$.

FIG. 12. Culture in flask of a fragment of an adult chicken thyroid (Experiment 8), which had been perfused for 8 days with a medium containing Witte's peptone. Photograph shows the colony of epithelial cells developed after 3 days' cultivation. $\times 115$.







(Carrel: Culture of whole organs. 1)





(Carrel; Culture of whole organs I)



EXPERIMENTAL NEPHRITIS IN RATS INDUCED BY INJECTION OF ANTI-KIDNEY SERUM

II. CLINICAL AND FUNCTIONAL STUDIES*

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The glomerulonephritis induced by anti-kidney serum has been the subject of extensive study. Recent workers in the field have emphasized the close similarity of this experimental disease to human nephritis. Our purpose in this paper is: (a) to present clinical and functional data on a group of rats which developed nephritis after treatment with anti-rat-kidney serum, and (b) to point to functional evidence of the progressive nature of the induced disease. Pathological observations on the majority of the animals reported here are given elsewhere (1).

Anti-kidney serum was first used by Lindemann (2) in 1900 and was studied by Pearce (3) in 1904. The earlier workers were primarily interested in the immunological and pathological aspects of the problem. Rats have been previously employed in experiments with nephrotoxic serum only by Masugi (4, 5), who limited his clinical studies of renal damage to urinalysis.

Moderate elevation of the blood pressure some time after treatment with anti-kidney serum has been recorded in dogs by Lüdke and Schüller (6) and in rabbits by Masugi (7), Arnott, Kellar, and Matthew (8), and Koráni and Hámori (9). Pearce (3) observed no immediate effect on the blood pressure of dogs and rabbits injected with nephrotoxic sera. Takeda is quoted by Masugi (5) as having found in rabbits a distinct rise in the non-protein nitrogen of the blood. In three of six rabbits Masugi (7) observed a steady rise of the blood non-protein nitrogen from the time of injection with nephrotoxic serum until the animals died or were sacrificed about 4 weeks later. One animal (No. 6) showed an increase in the blood non-protein nitrogen of from 50 mg. per cent, before injection, to 224 mg.

* Read in abstract before the American Society for Clinical Investigation, Atlantic City, May 4, 1936.

per cent several days prior to death. Kashiwabara's (10) data on the various non-protein nitrogen fractions of the blood and urine of rabbits, during a period of 2 weeks' observation after receiving anti-kidney serum, showed no significant change.

Methods and Materials

Anti-rat-kidney serum was prepared in rabbits by immunization with suspensions of perfused rat kidney. The preparation of nephrotoxic serum 4138, used throughout these experiments, has already been described (11). Young black and white hooded rats of both sexes from an inbred strain were used. The animals were given bread and dog biscuit daily, lettuce or cabbage and carrot twice weekly, and water *ad lib.* Vitamins were supplied in the form of wet baker's yeast and cod liver oil once a week.

Urea clearances were done by the procedure described by Farr and Smadel (12). Urine and blood for other studies were obtained by the methods used in the clearance determination. Blood urea nitrogen values were determined gasometrically by the hypobromite micro method of Farr (13). Quantitative urine albumin values were obtained by the method of Shevky and Stafford (14). The guaiac test for occult blood was done as routine on urine specimens and the urinary sediment was examined microscopically for formed elements. Plasma proteins were determined gasometrically by Van Slyke's micro Kjeldahl method (15). Plasma lipid values were obtained by the gasometric method of Kirk, Page, and Van Slyke (16). The gasometric micro procedure of Van Slyke and Sendroy was followed for blood calcium (17). Urinary chloride was determined by a modified Volhard-Harvey titration, as described by Peters and Van Slyke (18). The hemoglobin content of the blood was found by Sahli's method. Blood pressure was estimated with the apparatus of Moberg (19) but several changes in his technique were instituted. Pressures were determined on unanesthetized rats, for it was found that, after training, the animal remained quietly in the hands of an assistant throughout the procedure. The blood pressure was recorded at the point where blood again flowed through the previously compressed ear arteriole. This technique gave readings closely approximating the diastolic blood pressure. The pupil was dilated by homatropine for eyeground examination.

EXPERIMENTAL

A group of 18 young rats, weighing between 50 and 75 gm., was observed for a control period during which urea clearances, blood hemoglobin and plasma protein determinations, and urinary examinations were carried out. They were then divided into three subgroups as follows:

Group 1.—Ten rats. Eight of these (N-36 to N-43, inclusive) were treated with a total of 0.3 cc. per 100 gm. body weight of anti-kidney serum, given in divided amounts over a period of several weeks. Two other rats (N-78 and N-79) received a total of 0.45 cc. per 100 gm. body weight of serum in two injections on consecutive days.

Group 2.—Two rats (N-74 and N-75) were treated with a total of 0.65 cc. per 100 gm. body weight of anti-kidney serum, given in four divided amounts at 4 day intervals.

Group 3.—Six control rats. Of these, two (N-80 and N-81) were untreated; two (N-82 and N-83) received the serum of a rabbit immunized with rat serum; and the remaining two (N-76 and N-77) received anti-kidney serum completely absorbed by rat kidney or liver.

A fourth group of 29 rats had no chemical studies before treatment with nephrotoxin, and was subjected to less intense study after the induced disease was established. Rats N-21, N-22, N-24, N-25, and N-57 were of especial interest.

The examinations made during the control period were repeated at intervals after injection until the animals became moribund or were sacrificed. Plasma lipids and urinary chlorides were determined in a few instances. Blood pressure readings were obtained on a number of animals.

Urea Clearance.—The administration of anti-kidney serum in moderate amounts, that is, just sufficient to induce a marked albuminuria, cylindruria, and anasarca, usually had no immediate significant effect on the urea clearance, even though it became depressed at a later date. This is illustrated by rats N-37, N-39, and N-79 (Charts 1, 2, and 3) that received a series of small injections of anti-kidney serum, and by rat N-21 (Table I) that was given approximately the same total amount of nephrotoxin in a single injection.

The urea clearance fell rapidly and the animals died in apparent renal failure within a few weeks if a relatively large amount of nephrotoxin was given in several divided doses at short intervals (rats N-74, Chart 3, and N-75, Table III). The kidney damage in these animals was attributed to nephrotoxin alone, whereas another factor was present in rat N-78, Table III, which showed a similar rapid depression of the clearance. This rat had an anaphylactoid reaction following the first injection of serum and showed glomerular fibrin thrombi postmortem. In other studies (1, 11) severe anaphylactoid reactions were frequently associated with subsequent development of glomerular thrombi. Clearance studies were not done on these animals.

A progressive irreversible decline in the urea clearance resembling that in chronic active nephritis of man, was observed in certain animals that survived the acute experimental nephritis. Four rats (N-21, N-36, N-37, and N-39) became moribund, with apparent renal failure, from 84 to 313 days after treatment. The terminal clearance value on rat N-21 (Table I) was 3.6 cc. on the 84th day, while rats N-37

and N-39 (Charts 1 and 2) which died on the 313th and 240th days respectively, had final clearances of only 0.9 cc. per square meter per minute (normal 10.9 ± 3.1 cc. per square meter per minute (12)). Although a clearance value was not obtained terminally on rat N-36, its blood urea was 97 mg. per cent. Definitely progressive glomerular

TABLE I

Blood Urea Nitrogen, Urea Clearance, and Plasma Protein Values of Two Rats with Chronic Progressive Nephritis

Rat	Date	Blood urea N	Urea clearance	Plasma protein
	1935	mg. per 100 cc.	cc. per sq.m. per min.	gm. per 100 cc.
N-21	Feb. 20	6.7	33.8*	
Injected with:	Mar. 9	12.4	20.7	4.53
Anti-kidney serum	Apr. 10	16.6	11.2	
0.25 cc. Feb. 2	" 26	55.7	3.6	3.18
Total = 0.25 cc. per 100 gm.	" 27	Died		
N-36	Mar. 6	9.1	11.9	
Injected with:	" 12	9.0	17.2	
Anti-kidney serum	" 17	10.3	20.3	8.43
0.10 cc. Mar. 5 and 27	" 29	21.2	10.8	
0.025 cc. Apr. 23	Apr. 10	9.8	25.9	
0.075 cc. Apr. 24	" 19	9.3	15.6	
Total = 0.30 cc. per 100 gm.	" 29	12.3	13.9	
	May 12	40.9	6.0	
	" 19	10.1	16.0	
	" 30	25.7	6.8	5.46
	June 6	18.3	8.0	
	July 21	97.0		

Anti-rat-kidney serum, used throughout, was administered intravenously. The dosages are in cc. per 100 gm. of rat body weight.

* This clearance was done while the technique of determining the urea clearances was being developed. These high values became rarer with experience. For possible explanation of these variations, see Reference 12.

involvement was observed on histological examination of the kidneys of these four animals, and is described elsewhere (1). Seven other rats, N-38, N-41, N-43 (Table I), N-22, N-24, N-57 (Table II), and N-79 (Chart 3), that survived a severe acute nephritis, were followed for an average of 220 days, and were sacrificed from 171 to 225 days

after injection. In all of this group the urea clearances fell within the normal range when the observations were discontinued, although six animals still showed marked albuminuria and cylindruria.

TABLE II

Blood Urea Nitrogen, Urea Clearance, and Plasma Protein Values of Two Rats with Chronic Nephritis

Rat	Date	Blood urea N	Urea clearance	Plasma protein
	1935	mg. per 100 cc.	cc. per sq m. per min.	gm. per 100 cc.
N-43	Feb. 27	10.5	15.5	5.89
Injected with:	Mar. 16	6.2	46.0	
Anti-kidney serum	" 12	10.5	12.0	
0.19 cc. Mar. 15	" 17	8.4	22.9	
0.035 cc. Mar. 27	" 30	16.6	13.4	5.73
0.05 cc. Mar. 28	Apr. 10	20.0	14.3	
Total = 0.275 cc. per 100 gm.	" 19	15.9	12.9	
	" 26	11.8	17.7	
	May 12	35.2	8.1	
	" 19	39.1	6.0	
	" 30	23.4	9.6	6.51
	June 6	16.9	10.1	
	Aug. 5	18.0	11.4	6.82
	" 20	16.3	9.6	
	Sept. 5	30.5	6.4	
	" 18	12.0	13.3	6.39
	Oct. 11	20.6	11.4	
	Nov. 14	17.1	10.8	
N-57	June 7	11.4	14.2	
Injected with:	Aug. 6	14.2	8.2	5.74
Anti-kidney serum	" 20	14.6	7.5	
0.52 cc. Apr. 20	Oct. 11	13.3	10.3	
Total = 0.52 cc. per 100 gm.				

These animals failed to show a depression of clearance although albuminuria and cylindruria continued throughout the course of the disease.

The control rats in group 3 maintained normal clearance values throughout the period of observation, from 6 to 14 months (rat 80, Chart 4).

Blood Urea Nitrogen.—The animals in group 1, that received 0.3 cc. of anti-kidney serum, developed clinical signs of nephritis but did

not show significantly elevated blood urea nitrogen during the acute phase. On the other hand, the two rats in group 2 that received larger amounts of anti-kidney serum and died during the acute phase showed a rapid rise in the blood urea nitrogen; in rat N-74 (Chart 3) it reached 144 mg. per cent terminally. The blood urea nitrogen values rose as the urea clearance fell in the animals with chronic progressive lesions. The highest blood urea nitrogen value recorded in these studies was 312 mg. per cent, obtained terminally on rat N-37 (Chart 1). An unexpected lability of the blood urea nitrogen was encountered in certain rats which showed marked transient elevations of urea. These elevations were apparently due, not to acute renal crises but to such extrarenal factors as diarrhea (12), prostration, or circulatory insufficiency. The wide fluctuations of blood urea clearance during the last few months of life in rat N-37 (Chart 1) may have been due to vascular accidents with transient circulatory disturbance, since microscopic examination revealed degenerative lesions of different ages in the heart and other viscera (1).

Proteinuria.—Proteinuria became manifest within a few hours after the injection of anti-kidney serum, and for a time usually maintained a level of about 40 mg. per cc. Some animals, *i.e.*, rat N-79 in Chart 3, showed as much as 60 mg. of protein per cc. During the early phase of the acute nephritis a marked oliguria was present. This oliguria usually disappeared after several days; the protein in the urine remained, but its concentration fell to lower levels of 20 to 30 mg. per cc. Rats with chronic nephritis, both progressive and latent, as judged by the urea clearance and histological studies, generally excreted during 12 hours about 1.5 to 2.0 cc. of urine when water alone was given. The protein content of this urine was about 20 mg. per cc. Rat N-79 (Chart 3) was an exception to this generalization, as its urine became normal 6 weeks after injection. Rat N-37 (Chart 1), with slowly progressing nephritis, had a definite polyuria during the last months of life, averaging 6 to 7 cc. of urine for 12 hours. The protein concentration in this urine was less (5 to 10 mg. per cc.) during the terminal phase, but the total protein loss per day was approximately the same as earlier in the course.

Urinary Sediment.—Casts appeared in the urine about the 3rd day, usually in very large numbers. Hyalin casts were generally observed

first and were followed within a few days by granular and cellular casts. Cylindruria later became less intense, but persisted until death, or, in those animals which apparently recovered, until the proteinuria ceased. In only one rat, N-78, was there sufficient increase in erythrocytes in the urine to give a positive guaiac test; for the serum was administered in a manner to avoid, as far as possible, glomerular thrombosis and hematuria (1, 11). Doubly refractile globules were searched for on several occasions but were not encountered.

Edema.—Subcutaneous edema and ascites appeared about the 5th day after the injection of an adequate dose of nephrotoxic serum. The anasarca rapidly reached a maximum and remained severe for 4 or 5 days, although in certain instances it persisted for as long as 3 weeks. The majority of the animals then developed severe diarrhea, which was followed by complete elimination of edema fluid. The remaining animals with anasarca lost their edema after a transient period of diuresis. Lipemia usually appeared when the edema was marked. Two edematous rats had total blood lipid carbon values of 2589 mg. per cent and 964 mg. per cent, respectively, whereas the corresponding determinations in two normal animals were only 556 mg. per cent and 562 mg. per cent. Ascitic fluid from the first animal contained 63.5 mg. per cent of lipid carbon. There was no apparent failure to excrete chloride during this phase, since two rats with edema eliminated NaCl at the rate of 7.5 gm. per liter of urine, while a normal rat on the same diet excreted 9.25 gm. per liter.

Anemia.—Rats with acute nephrotoxic nephritis maintained a normal blood hemoglobin value of 70 to 80 per cent (Sahli). Only one animal with chronic nephritis, N-37 (Chart 1), developed anemia, and this did not appear until 8 months after injection. The hemoglobin rapidly fell throughout the last 6 weeks of life to a terminal value of 20 per cent. No other rat with chronic progressive lesions survived as long as this one; hence it is impossible to state whether anemia would have developed frequently in prolonged cases.

Hypertension.—Elevated blood pressure occurred only in the rats which developed chronic progressive nephritis. Early in the work several methods for determining the blood pressure were used without success, and only later was Moberg's technique employed. With it rats N-37 and N-39 (Charts 1 and 2) were shown to develop hyper-

tension in the 8th month of the disease. The recordings in these two rats steadily rose from a normal range of 50 to 60 mm. of Hg to slightly above 100 mm. where they remained until just prior to death, when a moderate drop was noted. Other animals of the same age, both controls and rats with latent nephritis, failed to show a rise in blood pressure. Hypertension was not observed during the initial phase of this induced disease (rat N-74, Chart 3).

TABLE III

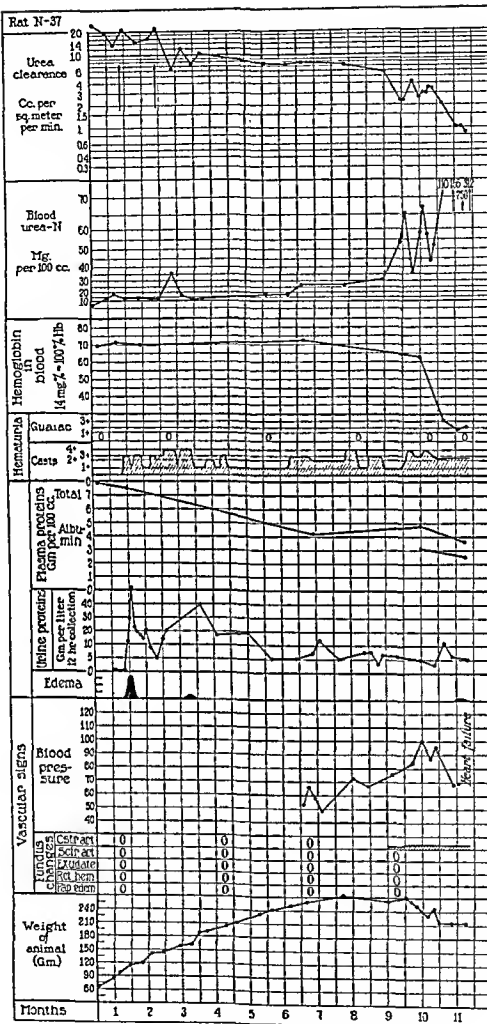
Blood Urea Nitrogen, Urea Clearance, and Plasma Protein Values of Two Rats Dying with Acute Nephritis

Rat	Date	Blood urea N	Urea clearance	Plasma protein
	1935	mg. per 100 cc.	cc. per sq.m. per min.	gm. per 100 cc.
N-75	May 12	8.6	38.5	
Injected with:	" 17	16.9	19.4	
Anti-kidney serum	" 22	19.3	15.0	
0.30 cc. May 8	" 26	47.7	5.8	
0.15 cc. May 23 and 27	" 30	36.2	6.7	
Total = 0.60 cc. per 100 gm.	June 3	68.3	2.7	
	" 4	165.3		6.81
N-78	May 17	12.4	23.0	
Injected with:	" 22	28.0	9.1	
Anti-kidney serum	" 25	24.6	8.9	
0.30 cc. June 10	June 3	17.6	13.3	
0.15 cc. June 12	" 17	50.5	5.3	
Total = 0.45 cc. per 100 gm.	" 23	142.6	0.9	4.63

N-75 illustrates a typical severe acute nephrotoxic response. The chemical and functional data on N-78 are similar; this animal, however, had in addition an anaphylactoid type of reaction with hematuria and thrombosis of glomerular capillaries.

Retinopathy.—Neither hemorrhage, exudate, nor papilledema was seen in any of the animals. Some constriction of the retinal arteries seemed to be present in rat N-37 throughout the last month of life. The marked anemia at that time, however, made the accurate visualization of the retinal bed difficult.

Plasma Proteins.—Although no systematic study of the plasma proteins was carried out, sufficient observations were made to indicate the general trend. Early in the acute phase of the induced disease, when the animals were losing large quantities of protein in the urine,



Nephrotoxin injected: Mar 15 0.10 cc. per 100 gm. iv
 " 27 0.10 " " " "
 Apr. 23 0.025 " " " "
 " 24 0.075 " " " "
 Total 0.30 " " " "

CHART 1

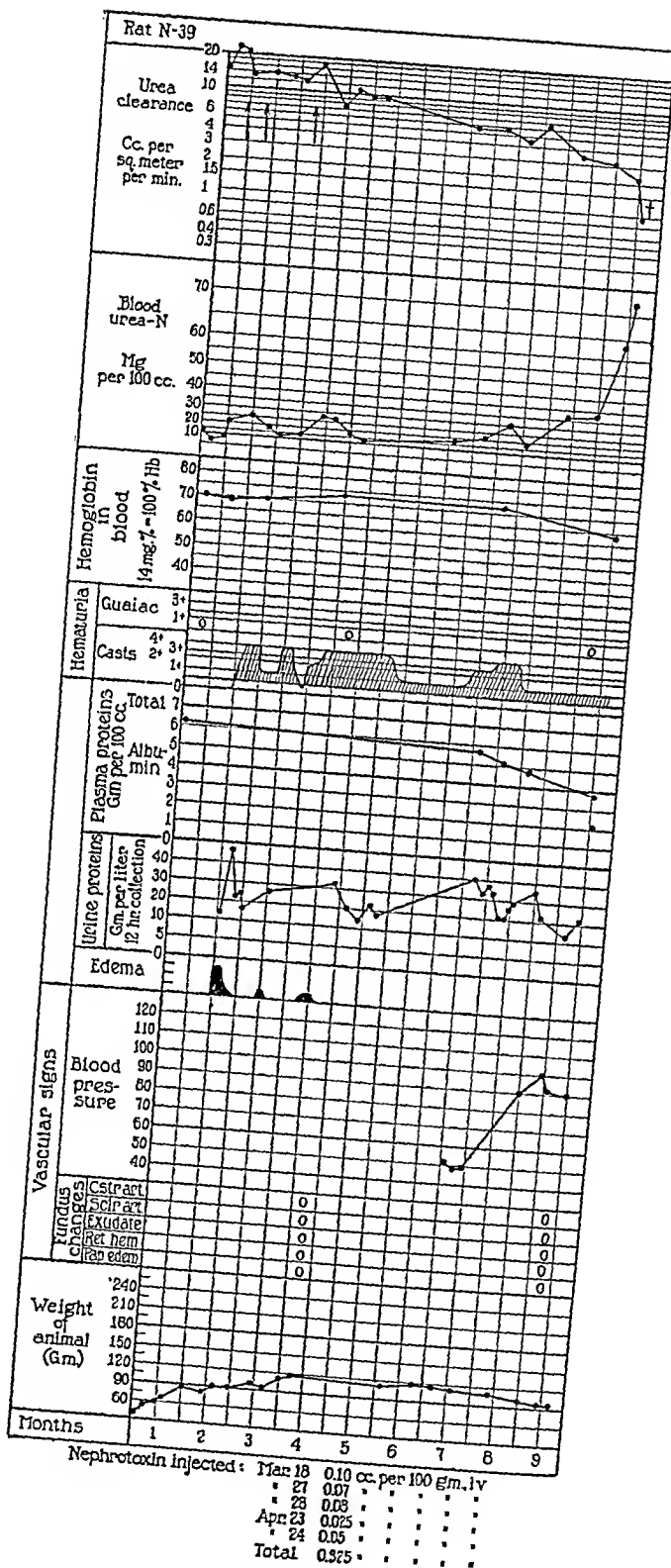
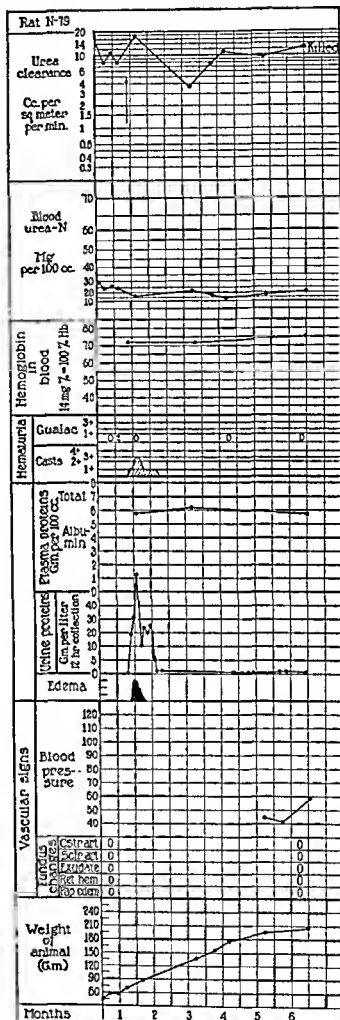
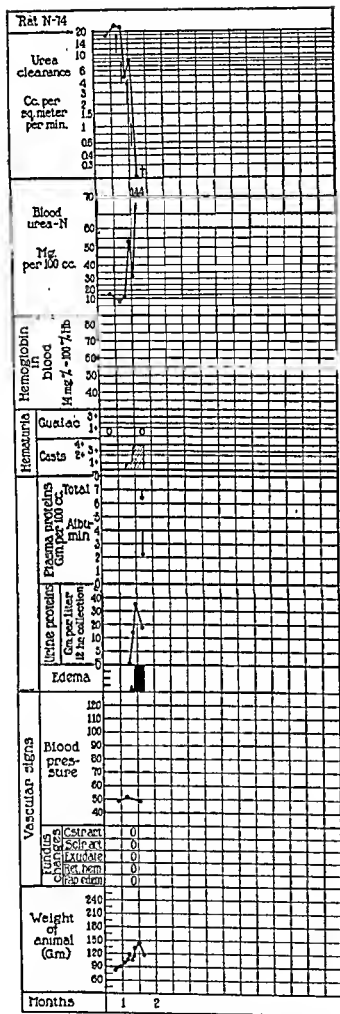


CHART 2



Nephrotoxin injected: May 18 0.30 cc. per 100 gm. i.v.
 • 23 0.15
 • 27 0.15
 • 31 0.075
 Total 0.675

Nephrotoxin injected: June 10 0.30 cc. per 100 gm. i.v.
 • 12 0.15
 Total 0.45

CHART 3

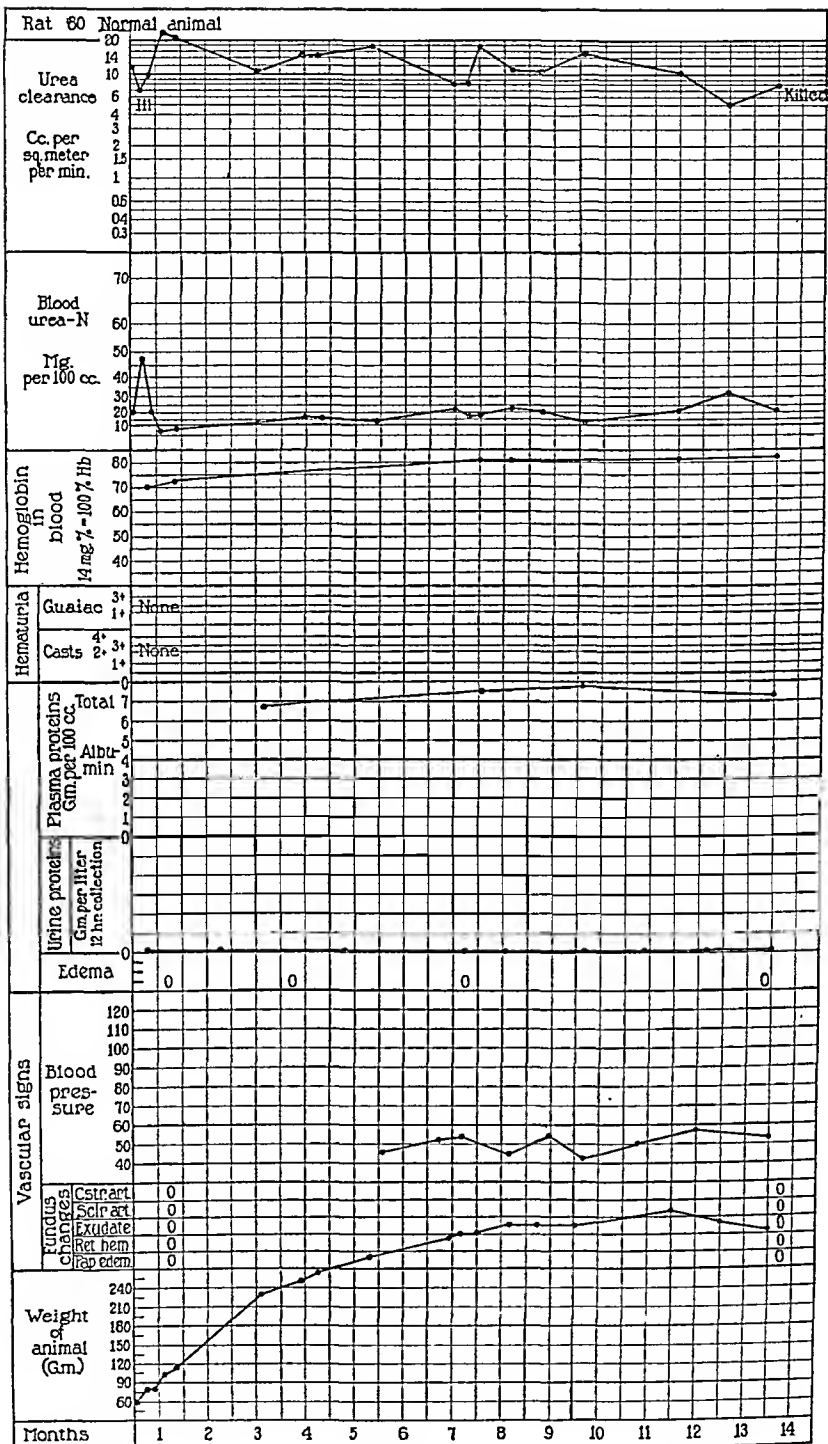


CHART 4

the plasma proteins were decreased from the normal of 6.5 per cent (20) to 4.51 per cent and 4.63 per cent in rats N-38 and N-78 (Table III), respectively. This fall in plasma protein was not a constant feature of the early disease, since N-74 when moribund on the 14th day had a total protein of 6.4 per cent with 2.2 per cent albumin. Animals with chronic nephritis had normal plasma proteins 2 months after treatment. Protein determinations were obtained during the terminal phase on two of the four rats with progressive nephritis (N-37 and N-39) and in both instances a depression was noted. Total plasma protein values of 3.58 per cent and 3.93 per cent were found, with albumin fractions of 2.5 per cent and 2.1 per cent, respectively. Animals that failed to develop progressive nephritis (N-22, N-24, N-38, N-41, N-43, N-79) had normal amounts of plasma protein when sacrificed after 6 to 8 months.

While plasma protein deficit depended in part, at least, on the large amount of protein excreted in the urine, synthesis of proteins also appeared important, since many of the rats which maintained normal plasma proteins lost as much protein in the urine as did those which developed a plasma protein deficit.

Growth.—Growth was permanently retarded only in certain of the animals with chronic progressive nephritis (rat N-39, Chart 2). In the initial phase, just after edema fluid had been eliminated, the animals usually appeared malnourished, and often weighed less than before the injection of anti-kidney serum. After a variable delay, growth was resumed and attained a normal range in most animals.

The cause of the retardation or the cessation of growth in the rats with severe renal involvement was not apparent. The possible occurrence of a process similar to renal rickets was considered; and rat N-39 was investigated with this in view. Blood calcium and phosphorous determinations, x-ray examination of epiphyses of long bones, and histological study of these bones revealed no significant abnormalities. The terminal weight loss observed in rats N-39 and N-37 was the result of malnutrition dependent upon marked anorexia.

SUMMARY

The glomerulonephritis induced in rats by nephrotoxin was characterized clinically during its initial phase by severe albuminuria, cylindruria, and anasarca, but not by hematuria.

Rapidly fatal nephritis was produced by injecting relatively large amounts of anti-kidney serum at frequent intervals. In such cases the blood urea mounted rapidly; the urea clearance fell; and death occurred within about 2 weeks.

A milder nephritis of the chronic type was induced by giving smaller quantities of anti-kidney serum in either single or divided doses. In these instances there was no immediate alteration of the urea clearance. Lipemia and plasma protein deficit appeared with the development of anasarca. The majority of rats which survived the initial stage of this experimental nephritis continued to show marked albuminuria with casts until they died or were sacrificed months later. Some of these animals showed retardation of growth and a progressive fall of the urea clearance. Terminally there developed marked retention of urea, plasma protein deficit, anemia, and hypertension.

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EXPERIMENTAL NEPHRITIS IN RATS INDUCED BY INJECTION OF ANTI-KIDNEY SERUM

III. PATHOLOGICAL STUDIES OF THE ACUTE AND CHRONIC DISEASE*

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PLATES 20 TO 22

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Antiserum prepared by immunizing rabbits with a suspension of perfused rat kidney contains an antibody relatively specific for rat kidney. Data supporting this conception were recorded in the first article of this series (1). The purpose of this communication is two-fold: to give in greater detail the histopathological picture of the developing lesions and to correlate the type of lesions with the clinical and renal functional changes previously reported (2).

Materials and Methods

Young black and white hooded rats weighing from 50 to 100 gm., of an inbred strain, were injected with unabsorbed anti-rat-kidney serum, No. 4138. The preparation and action of this serum are detailed in another communication (1).

The clinical and functional observations on most¹ of the animals mentioned below were reported in the preceding paper (2), and the rats are designated in the same way in both communications. The blood urea determinations were done by the micro method of Farr (3) and the urea clearance values were obtained by the technique of Farr and Smadel (4).

Rats were sacrificed at intervals after injection of anti-kidney serum or when moribund. Autopsy was performed immediately and portions of organs were fixed in 5 per cent Zenker's acetic solution and in 10 per cent neutral formalin. Paraffin sections of various organs were stained as routine with methylene blue-

* Read in abstract before the American Association of Pathologists and Bacteriologists, Boston, April 9, 1936.

¹ Chemical determinations in a few of the animals, not recorded in the previous paper, were obtained through the kindness of Dr. Lee E. Farr.

eosin. In addition, Mallory's connective tissue stain and McGregor's modification (5) of the Mallory-Heidenhain stain were applied to sections of all kidneys. Fatty changes, when present, were shown in frozen sections stained with Scharlach R-hematoxylin. Other methods were occasionally used.

Acute Nephritis Induced by the Injection of Anti-Kidney Serum

The syndrome induced by the relatively specific nephrotoxin, was characterized clinically by marked and persistent albuminuria, cylindruria, and transient anasarca; significant hematuria did not occur (1). Macroscopic observation of the kidney at this stage revealed a moderately pale mottled smooth surface. The cortex bulged slightly on section, the glomeruli were visible as tiny pin-point elevations. Microscopically, the glomerular tufts were relatively anemic, yet they appeared large and almost filled the capsular spaces. This fullness of the tuft depended principally upon thickening of the capillary walls, which stained brightly with eosin and gave a refractile effect. The thickening of the glomerular capillary walls was best seen in sections stained with azan carmine (Mallory-Heidenhain technique) which McGregor (5) considered specific for the glomerular capillary basement membrane. This thickening of the capillary walls was roughly proportional to the amount of nephrotoxin administered, and varied to some extent throughout each tuft and to a greater extent among different tufts. This variation was most noticeable after a single moderate sized dose of nephrotoxin, and was least apparent when a relatively large amount of anti-kidney serum was given in several fractional doses. A number of the tuft nuclei and capsular cells were swollen. Proliferative changes in the glomeruli were inconspicuous, and infiltration of inflammatory cells into the tuft was practically non-existent. Fatty degeneration did not occur in the cells of either glomerular tuft or capsule during the first few days. In the latter part of the acute stage, however, a few cells in each glomerulus, usually epithelial cells, contained fat droplets.

The epithelial cells of the majority of the convoluted and straight tubules, especially the former, were the seat of cloudy swelling and of hyalin droplet degeneration. The lumina of many tubules were moderately distended with a granular protein precipitate. Scattered groups of tubules in cross section, apparently belonging to a single nephron, had widely dilated lumina often filled with granular material

or casts, and were lined by low cuboidal epithelium with slightly basophilic cytoplasm. Fatty degeneration was not observed in the tubular cells during the 1st week after injection, but usually occurred in a moderate degree later in the acute stage of the disease.

Collections of cells were frequently present about small vessels in the kidney, generally in the cortex. These were composed of mononuclear cells, lymphocytes, and eosinophilic polymorphonuclears; they appeared about the 4th day of the induced disease and increased somewhat thereafter. Indefinite changes occurred in the small arteries and arterioles; there was some swelling of the endothelial nuclei and slight hyalinization in the muscle coat. Microscopic alterations in other organs were limited to a perivascular reaction similar to that in the kidney, but less extensive.

Rat N-35, male, weighing 87 gm., was injected intravenously with 0.25 cc. per 100 gm. body weight of the nephrotoxic serum, No. 4138. A slight anaphylactoid reaction developed within a few minutes and subsided after a quarter of an hour. Urine collected during the first 18 hours contained over 4 per cent protein but no blood. Numerous casts appeared the next day. A blood urea nitrogen value of 34 mg. per cent was obtained on a specimen taken when the animal was sacrificed 48 hours after injection. Postmortem examination revealed 4.0 cc. of clear straw-colored ascitic fluid. The appearance of the kidney was as described above. Abnormalities of both glomeruli and tubules were less intense than in rat N-34 which received the same treatment but was sacrificed 2 days later. The clinical course of this second animal resembled that of the first but its terminal blood urea nitrogen reached 49 mg. per cent. Figs. 1, 2, and 3 depict the renal lesions of rat N-34.

Had these two animals (N-34 and N-35) not been sacrificed they would undoubtedly have survived the acute phase of the disease, as the dose of nephrotoxic serum was not large. Indeed, two or three times this dose was usually required to cause death during the acute phase. When this larger amount of serum was administered in divided doses, so that none induced a severe anaphylactoid reaction, then the clinical picture was as described above except that the blood urea rose to greater heights. The pathological alterations were qualitatively similar but quantitatively greater.

Rat N-74 received a total of 0.65 cc. per 100 gm. body weight of serum 4138 in four divided doses given at 3 day intervals. When moribund on the 16th day

its blood urea nitrogen value was 144 mg. per cent. Fig. 4 demonstrates the marked uniform swelling of the glomerular intercapillary material throughout the tuft.

When a severe anaphylactoid reaction was induced with anti-kidney serum, either as a result of giving a comparatively large amount of a relatively pure nephrotoxic serum or a smaller amount of serum rich in non-organ specific anti-rat-tissue antibodies as well as in the more specific nephrotoxin, the clinical and pathological picture differed (1) from that just described. Hematuria with death, occurring from a few hours up to 8 days, were the principal clinical differences. The animals thus affected showed many fibrin thrombi in the glomerular tuft capillaries; and often in addition there were lesions of vascular origin in other organs similar to those observed in anaphylactic shock in rats (6). The protocol given below (rat N-18) is that of an animal which had a rather severe anaphylactoid reaction after injection of a considerable amount of relatively pure nephrotoxic serum.

Rat N-18, weight 106 gm., received 0.51 cc. of serum 4138 in a single injection. Marked albuminuria and gross hematuria appeared within 12 hours. Albuminuria was constant until death on the 8th day, whereas hematuria decreased after the 1st day and was then made evident by a faintly positive guaiac reaction for the next 4 days. Numerous casts appeared on the 3rd day, when slight subcutaneous edema and ascites were noted. The anasarca became marked and the body weight rose to 134 gm. on the 6th day. The terminal blood urea nitrogen value was 156 mg. per cent. At autopsy extensive anasarca was noted. The kidneys macroscopically were moderately swollen, edematous, and pale.

Microscopic study revealed that portions of the capillary loops in the majority of the glomerular tufts were filled with thrombi (Fig. 5). These thrombi, colored purple by Weigert's fibrin stain, varied in size from a thin strand along one side of a capillary to a large sausage-shaped mass completely filling the space between the walls. In some instances the thrombus extended out into the afferent arteriole. The extensively thrombosed glomerular tufts showed necrosis of cells in patchy areas and in some instances granular protein precipitate or erythrocytes were seen in the capsular space. Thickening of the glomerular capillary basement membranes was best seen in tufts without thrombi. The tubular changes were similar to those observed when the effect was induced by the nephrotoxic action of the serum alone, uncomplicated by the added anaphylactoid reaction.

Chronic Nephritis Occurring after the Injection of Anti-Kidney Serum

The transition from the acute to the chronic phase of the nephritis induced by anti-kidney serum was indefinite clinically. If not too

severely injured, the majority of animals recovered from the edematous state and continued to show a moderate to marked cylindruria and albuminuria until they became moribund or were sacrificed. The urea clearance determination in these animals (2) usually did not indicate decreased renal function until several months after injection of the nephrotoxin. By histological criteria the kidneys had generally become extensively damaged before the urea clearance fell to what we have considered as a significantly low range. None of the rats that passed through the acute phase of the experimental nephritis (arbitrarily set at 21 days (2)) became moribund before the 84th day.

In order to study the pathological changes during this intermediary period, seven rats were sacrificed between 30 and 40 days after receiving an amount of nephrotoxic serum adequate to induce severe acute nephritis. There was little divergence in the clinical course or the pathological findings of these animals. The findings in rat N-25 were typical.

Microscopic Examination of Kidney.—Rat N-25. The histological lesions observed in kidneys of rats with the acute disease were present together with certain additional features. Many of the glomerular tufts were distorted; a few had club-shaped loops, and occasionally a significant increase in nuclei was noted in a portion of a tuft. Proliferation of capsular epithelial cells, forming a small crescent, was occasionally encountered. This was usually accompanied by a thickened hyalinized glomerular capsular membrane which was surrounded by lymphocytes and fibroblasts. In several areas the tubular basement membranes were thickened and the interstitial connective tissue was increased. Changes in the arteries were indefinite.

The chronic stage was studied in eleven rats that survived a severe acute nephritis. These animals were followed for periods varying from 84 to 313 days after injection; the average for the group was 208 days. Four of the eleven were killed when moribund. The others looked healthy when sacrificed, although all but one still showed albuminuria and casts. The most significant histological findings in these chronically affected animals were those indicating definite progression of the disease process as shown by glomerular crescent formation and scarring of different ages which occurred in the same microscopic field. In addition, cystic dilatation, atrophy, and hyalin droplet degeneration of the tubules were quite extensive.

Detailed studies are given of several of the eleven animals followed for from 3 to 10 months after treatment.

Rat N-21 received a single injection of 0.25 cc. per 100 gm. body weight of nephrotoxic serum 4138. At autopsy on the 84th day it had some subcutaneous edema and ascites. Another animal, N-36, received a total of 0.3 cc. of the same serum, given in fractional amounts over a period of 7 weeks, and was killed on the 98th day after the last injection, when markedly prostrate and while having intermittent generalized convulsions and muscular tremors. Both of these rats had developed slight edema during the acute phase and showed persistent albuminuria and cylindruria. The terminal blood urea nitrogen determinations were 55 and 97 mg. per cent, respectively. Cultures of the blood and of the kidneys taken at autopsy were negative in both animals; moreover, serum of neither contained agglutinins for *Bacillus enteritidis*. The macroscopic and microscopic descriptions of the organs of the two animals varied in minor respects. The protocol of rat N-36 is given below, since more complete chemical studies were made on the blood and urine of this animal (2).

Autopsy.—Rat N-36, female, weight 128 gm. The kidneys appeared enlarged and weighed 1.1 gm. each. The capsule stripped easily, revealing a pale surface which was roughly granular with many elevations of yellowish tan kidney substance as well as tiny cysts. On section, the cortex was of the normal thickness but was poorly demarcated from the medulla. The pale medulla contained many markedly dilated tubules filled with coagulated material, which in cross section gave a honeycomb appearance, and in longitudinal section a marked radial streaking. The other organs showed no significant macroscopic changes.

Microscopic Examination of the Kidney.—Most of the glomeruli were abnormal. Some had widely dilated glomerular spaces and small tufts, but the majority were large with tufts that almost entirely filled the spaces. The glomerular capillary walls were hyalinized and irregularly thickened; the Mallory-Heidenhain stain again showed this to be due principally to thickening of the intercapillary material. Clubbing of capillary loops occurred, and usually one or more loops appeared to have an increase in nuclei. Pyknosis and karyorrhexis of tuft cells were present but not conspicuous. The basement membrane of the capsular epithelium was generally thickened and hyalinized. The majority of the glomeruli were the seat of varying degrees of crescent formation and scarring. A number showed the same type of tuft changes observed above, with the addition of proliferation of the capsular epithelial cells. Mallory's connective tissue stain failed to show blue fibrils in these particular crescents, while in other glomeruli, usually with more extensive capsular proliferation, an ingrowth of connective tissue fibrils into the crescent was visible. Still other glomeruli were in more advanced stages of scarring; the oldest had completely lost their normal architecture and were represented in the Mallory stained sections by concentric whirls of blue fibrils at the periphery and interlacing fibrils in the center. With Scharlach R stain numerous fat laden cells were visible in most of the glomeruli; those with marked crescent

formation often showed many fat droplets in the epithelial cells of Bowman's capsule as well as in the tuft; and rarely, in the latter position, large masses of fat occurred.

The tubular changes were extensive. The most prominent feature microscopically was the great number of large cyst-like tubules. These were in groups, most abundant in the corticomedullary region; in the cortex they often extended to the capsule, producing elevations. The dilated tubules were lined by flat, or occasionally low cuboidal, epithelium in good state of preservation and filled with hyalin material which took either acidophilic or basophilic stain. Thickening of the basement membranes of these dilated tubules was not demonstrable, nor was there any increase of interstitial connective tissue in these areas.

Another type of atrophic change was frequently met with in groups of convoluted tubules. These were smaller than normal; some had narrow empty lumina, but in most, the lumina were obliterated. The epithelial cells had prominent vesicular nuclei and relatively scanty basophilic cytoplasm. Thickening and hyalinization of the tubular basement membrane was present. Between the tubules was a varying amount of cellular interstitial tissue containing connective tissue cells, lymphocytes, and an occasional eosinophilic polymorphonuclear leucocyte. These scarred areas were scattered throughout the cortex, but were most common near the surface, adjacent to the elevations produced by the cystic tubules.

Finally, enlarged convoluted tubules were seen in nests in the outer cortex or in elongated radial groups in the inner cortex; these were lined by hypertrophic epithelial cells which projected in a dentate fashion into the moderately dilated lumina. The coarse cytoplasmic granules of these cells took a bright pink with methylene blue-eosin and brick red with Mallory's stain. The lumina were empty or loosely filled with pink granular material in which degenerated epithelial cells were sometimes seen. Thickening of the tubular basement membrane was inconstant and never extensive, nor was the interstitial tissue in these areas appreciably increased. Occasionally fat droplets were seen in the epithelial cells, usually in those of the neck of the tubules.

In addition to the alterations already described in the interstitial tissue, numerous eosinophilic polymorphonuclear leucocytes were scattered singly or in loose foci throughout the section. Vascular changes consisted of cellular collections in the adventitia of certain interlobular and arcuate arteries, swelling or pyknosis of nuclei in the media of larger arteries, and some thickening of the walls of arterioles.

Other Organs.—A similar picture was observed in the vessels of other organs, especially in the heart, brain, pancreas, and spleen. The perivascular infiltrations were less frequent and when encountered were much less marked, but the changes in the media of arteries and in the arterioles were about as definite. Several areas of scarring of different ages were present in the myocardium surrounding or adjacent to affected small vessels; moreover, in the cerebral cortex an area of typical encephalomalacia was found. Eosinophilic polymorphonuclears were

observed in all the organs. They were found in the mediastinal tissue, about the pulmonary vessels, and in the splenic pulp with greater than normal frequency. The only other significant finding was a moderate amount of chronic interstitial pneumonitis. Figs. 6 to 11 illustrate lesions in the kidney and heart of this animal.

Rats with even more slowly progressing nephritis, as indicated by a still later decline in the urea clearance, had lesions in the kidney and elsewhere similar to those observed in animals dying after 4 months, but showed more connective tissue replacement.

One such animal (N-39) in the chronic group became moribund 6½ months after the last injection of anti-kidney serum, and another (N-37) 9 months after. These followed much the same clinical course as N-36, and had terminal blood urea values of 72 and 312 mg. per cent respectively. The macroscopic organ changes of N-39 were practically identical with those observed at 3 to 4 months. Rat N-37 had a number of additional findings. The kidneys were more markedly enlarged, weighing 2.1 gm. each and measuring 30 x 15 x 7 mm. The cortex was more granular, with depressed scars approximately 1 mm. deep alternating with areas of kidney tissue several millimeters in width. The heart appeared definitely enlarged, with thickening of the left ventricular wall. Furthermore, the heart and liver gave macroscopic evidence of fatty change, and the testicles were much atrophied.

The microscopic picture of the kidneys of both these animals differed from rat N-36 essentially only in the greater extent of connective tissue replacement. Each of the lesions described in detail as occurring at 4 months was present at 6½ and 9 months. The glomeruli, in general, showed more extensive connective tissue ingrowth into crescents and tufts; still, fields were easily found in which the various stages of the process were represented, *i.e.* (a) thickening of the glomerular capillary basement membrane; (b) proliferation of the epithelial cells of Bowman's capsule with the tuft changes; (c) connective tissue ingrowth into the crescent; and finally (d) conversion of the entire glomerulus into a scar. There was a greater increase in interstitial connective tissue than at 4 months. Focal collections of cells were less frequent as the age increased; moreover, the eosinophilic polymorphonuclear foci had disappeared. Rat N-37, which had a terminal anemia of rapid onset, showed a greenish yellow pigment in the epithelium of the atrophic collapsed tubules in scarred areas, as well as in the spleen both intra- and extracellularly.

Another type of glomerular lesion, seen rarely in animals dying in 3 months, but consistently in those succumbing later, consisted of fibrin-like material in the crescents and tufts. This substance was often plentiful and appeared as thick homogeneous masses or finely granular clumps. It stained as does fibrin with methylene blue-eosin and Mallory connective tissue stains, but with Weigert's technique the appearance was less typical of fibrin. Glomeruli that were ap-

parently heavily laden with fibrin, as judged by the first two stains, showed with Weigert's stain a meager amount of purplish blue material arranged in loose thin strands with very fine blue dots scattered between. The impression was gained that this represented a degenerative change and not true fibrin deposit.

The arterial and arteriolar alterations described in rat N-36 were present in the kidneys of these older rats in about the same degree and severity. The vascular lesions in other organs, however, were more striking. The heart particularly was affected. The coronary arteries showed thickened walls with hyalinization, and in some instances calcium deposit in the media, as well as fraying of the internal elastic membrane. Fatty degeneration of the wall was demonstrable. The arterioles were also thickened. A focal fibrous myocarditis, secondary to the vascular lesions, was observed in rats N-37 and N-39, in all stages from fresh necrosis of groups of muscle fibers to the final complete replacement by fibrous connective tissue. The brain of rat N-37 had an area of encephalomalacia in the cortex, and in addition a large area of gliosis in the midbrain with diminution of ganglion cells. Figs. 12 to 14 depict lesions in the kidney, heart, and pancreas of rat N-39.

Another group of these chronically affected animals showed albuminuria and casts throughout the period of observation, but gave no evidence of progressive disease, such as failure to gain weight, development of hypertension, or significant depression of the urea clearance. These animals, N-22, N-24, N-38, N-41, N-43, N-57, were sacrificed from 190 to 260 days after injection. Most of the kidney abnormalities described in the animals N-39 and N-37 were demonstrable in sections from these rats, but the damage was less extensive, and actively progressing disease was not histologically evident except in rat N-57. This animal had the most markedly involved kidney of any in this group, with lesions almost as severe and as varied as in the severely affected rat N-39; even young glomerular crescents were present. It is probable that had this animal not been sacrificed so soon it would shortly have developed clinical signs of progressive nephritis. Rat N-79, on the other hand, which after going through a severe acute phase to a complete clinical recovery, had an almost normal histological picture when sacrificed 6 months later, and showed only very old scars involving occasional nephrons. In none of these animals was the vascular change outside the kidney of any import, and even in the kidney it was never as notable as in animals with clinically progressive disease. Myocarditis and encephalomalacia were not encountered, nor was there any other significant lesion except a frequent chronic pneumonitis.

DISCUSSION

The present study demonstrates that rats, injected with anti-rat-kidney serum, develop a glomerulonephritis which is, in certain animals, a progressive disease. During the acute phase there is microscopic evidence of injury to both the glomeruli and tubules. The former are enlarged and anemic, due principally to swelling and thickening of the glomerular intercapillary substance. The tubular epithelial cells undergo necrobiotic changes of which hyalin droplet degeneration is the most common. A number of tubules are dilated with hyalin casts. Thrombosis of glomerular capillaries does not result from nephrotoxin alone; but fibrin thrombi are conspicuous in the glomeruli when the effects of a severe anaphylactoid reaction (1) are added to the action of nephrotoxin.

The histopathological lesions of the chronic stage of the pure nephrotoxic nephritis develop gradually and take their origin from those occurring during the acute phase. Proliferation of the cells of Bowman's capsule is followed by connective tissue ingrowth and finally by complete replacement of the glomerulus by scar tissue. In animals with progressive decline of the urea clearance (2) different individual glomeruli are found, in the same microscopic field, with scarring of apparently different duration. The tubules are severely damaged; extensive dilatation with large hyalin casts, atrophy in areas of interstitial scarring, and finally hyperplasia of the remaining functioning units are all present. Generalized vascular lesions with secondary areas of degeneration in various organs occur only in the animals with progressive chronic nephritis.

The chief literature dealing with this type of induced nephritis has already been reviewed (1). While the present investigation discloses no new kidney lesions, it does differentiate the early histological effects of nephrotoxin from those of the non-organ specific tissue antibodies. It traces, in addition, the progressive nature of the chronic nephritis that originates in the acute damage induced by so called nephrotoxin. The fibrin thrombosis of glomerular capillaries, as described by Wilson and Oliver (7), Takeda (8), Masugi (9, 10), and Hemprich (11) in animals dead shortly after receiving anti-kidney serum is, in the case of rats, attributable to factors other than the relatively organ specific antibody nephrotoxin. Moreover, the prolif-

eration of glomerular endothelium in rabbits during the acute phase recorded by certain authors (12, 11, 16) has occurred only occasionally in our hands and then only after massive doses of anti-kidney serum. The data available are insufficient to determine whether the endothelial proliferation is attributable to the nephrotoxic or to the anapylactoid effect. The acute tubular lesions are similar to those described by others and are considered to be caused by the nephrotoxin. The swelling of the glomerular intercapillary substance in the kidneys of rats with acute nephrotoxic nephritis, brought out so clearly by the Mallory-Heidenhain stain, is apparently the same lesion that Masugi interprets as "condensation of the tuft wall" (9) or as "edema of the capillary wall" (12) and that Weiss (13) considers to be "serous exudate between the capillary walls."

The first convincing evidence of the induction of chronic nephritis with nephrotoxic serum was presented by Masugi (12), although others (14, 15, 8) noted glomerular crescent formation in a few animals observed for several months. Masugi (12) as well as Arnott and his coworkers (16) describe or give photomicrographs of rabbit kidneys, the seat of chronic experimental nephritis, in which individual glomeruli vary in the extent of their scarring; neither author, however, discusses differences in the duration of the disease processes which would connote progression of the nephritis. The development of chronic progressive nephritis is illustrated in the present paper by the records of two rats (N-21 and N-57) which received single injections of nephrotoxic serum, and also by three rats (N-36, N-37, and N-39) each of which received a series of several small injections of the same serum over a period of 7 weeks.

Worthy of special notice were the well marked generalized vascular lesions with areas of secondary degeneration in the heart, brain, and testes of rats with progressive chronic nephritis. That this vascular injury was dependent upon extensive renal damage is indicated by its failure to occur in normal rats of the same age, in rats receiving other antisera, or in rats which failed to develop clinical evidence of progressive nephritis after receiving anti-kidney serum. No mention of this type of generalized vascular lesion has been made by other authors. Cardiac hypertrophy, such as existed in rat N-37, has been occasionally noted by others (14, 12, 11).

A spontaneous glomerulonephritis occurring in rats has been regarded by Jaffé as a focal involvement (17). In our experiments approximately 460 rats have received either anti-rat-kidney serum or an antiserum against other rat tissues. In addition, 50 untreated animals were observed for from 2 months to a year. Among half of this last group, which was kept on a diet of bread and milk, several developed albuminuria and hematuria; at autopsy pyelonephritis was encountered and from the kidneys *B. enteritidis* was isolated. Certain untreated rats, without urinary abnormalities, had focal areas of chronic inflammation and scarring in the kidneys; and from the kidneys of these animals *B. enteritidis* was usually cultured. This type of infectious nephritis was encountered most frequently in the rats maintained on a poor diet and in rats obtained from one particular animal breeder. The black and white hooded strain of rats used in these experiments remained practically free from infectious nephritis when fed an adequate diet. The renal lesions attributable to anti-kidney serum in this communication were distinctly different from those observed in spontaneous infectious nephritis.

The clinical and functional studies recorded in the previous paper of this series (2) and the histopathological observations presented here indicate that a genuine diffuse glomerulonephritis is induced in rats by the injection of anti-rat-kidney serum, and that this experimental disease simulates, in most respects, glomerulonephritis in man. At present, however, it is hazardous to postulate that the mechanism responsible for nephrotoxic nephritis in laboratory animals is closely related to the etiology of human nephritis, and it is also unprofitable to compare too minutely the pathological changes in the acute experimental nephritis of laboratory animals with the lesions of the early human disease. Nevertheless, the close approximation of these two diseases offers an opportunity to obtain certain information from studies in animals that may be applicable to the clinically similar disease of man.

CONCLUSIONS

Administration of the relatively organ specific antibody, so called nephrotoxin, present in anti-kidney serum, is followed by a diffuse glomerulonephritis. This is characterized early by swelling of the

intercapillary substance of the glomerular tuft and by tubular degeneration. Fibrin thrombi are only present in the glomerular capillaries when the injection of anti-kidney serum results in a severe anaphylactoid reaction, and are due to factors other than nephrotoxin.

The urinary abnormalities which develop in all rats after a suitable injection of nephrotoxin usually continue until the animal dies or is sacrificed. Microscopic renal lesions of the early phase merge into scarring of the glomeruli and tubules. Histological study of those animals which die from 3 to 11 months after treatment reveals a chronic progressive glomerulonephritis with generalized vascular lesions.

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EXPLANATION OF PLATES

PLATE 20

FIG. 1. Rat N-34, male, weighing 87 gm., sacrificed 4 days after receiving 0.25 cc. per 100 gm. body weight, anti-rat-kidney serum (from rabbit 4138). Corticomedullary region. Methylene blue-eosin stain. The convoluted tubules about the glomerulus show hyalin droplet degeneration. Other tubules are dilated and have flattened epithelium; some have empty lumina while others are filled by hyalin casts or debris. Occasional epithelial cells are necrotic. $\times 125$.

FIG. 2. Rat N-34. Glomerulus. Methylene blue-eosin stain. The glomerular capillaries are almost free of erythrocytes, yet the tuft is large, due to thickening of the glomerular capillary walls and, to some extent, swelling of the tuft nuclei. $\times 450$.

FIG. 3. Rat N-34. Glomerulus. Mallory-Heidenhain stain. The deeply staining glomerular intercapillary substance or capillary basement membrane is clearly demonstrated. This thickening varies in different loops. $\times 450$.

FIG. 4. Rat N-74, female, weighing 108 gm., received 0.65 cc. per 100 gm. body weight of nephrotoxic serum (No. 4138) given in four divided doses at 3 day intervals. Animal died on 16th day. Glomerulus. Mallory-Heidenhain stain. The swelling of the glomerular capillary basement membrane is greater and more uniform than that in Fig. 3. $\times 450$.

FIG. 5. Rat N-18, male, weighing 106 gm., severe anaphylactoid reaction after receiving a single injection of 0.5 cc. per 100 gm. body weight of nephrotoxic serum (No. 4138). Died on 8th day. Glomeruli. Methylene blue-eosin stain. One glomerulus has numerous fibrin thrombi in the tuft capillaries, and cells in portions of loops are degenerated. The other glomerulus resembles Fig. 2. $\times 450$.

PLATE 21

FIG. 6. Rat N-36, received a total of 0.3 cc. per 100 gm. body weight of serum 4138 in four divided injections over a period of 7 weeks. Moribund 98 days after last injection. Corticomedullary region. Mallory connective tissue stain. Extensive tubular destruction of various types and interstitial scarring are evident. $\times 48$.

FIG. 7. Rat N-36. Cortex. Mallory-Heidenhain stain. Glomeruli in different stages of scarring are present. The several types of tubular lesions described in the text are illustrated. $\times 125$.

FIG. 8. Rat N-36. Glomerulus. Mallory-Heidenhain stain. A large glomerulus is shown with distorted tuft, irregularly thickened glomerular capillary basement membranes, and proliferated capsular epithelium into which connective tissue is beginning to infiltrate. $\times 450$.

FIG. 9. Rat N-36. Heart. Methylene blue-eosin stain. The upper vessel shows thickened and increased cellularity of the media and a loose infiltration of the cells in the adventitia. $\times 400$.

PLATE 22

FIG. 10. Rat N-36. Kidney. Scharlach R-hematoxylin stain, frozen section. Two glomeruli have dark staining masses representing fat, while a third tuft contains no fat. $\times 110$.

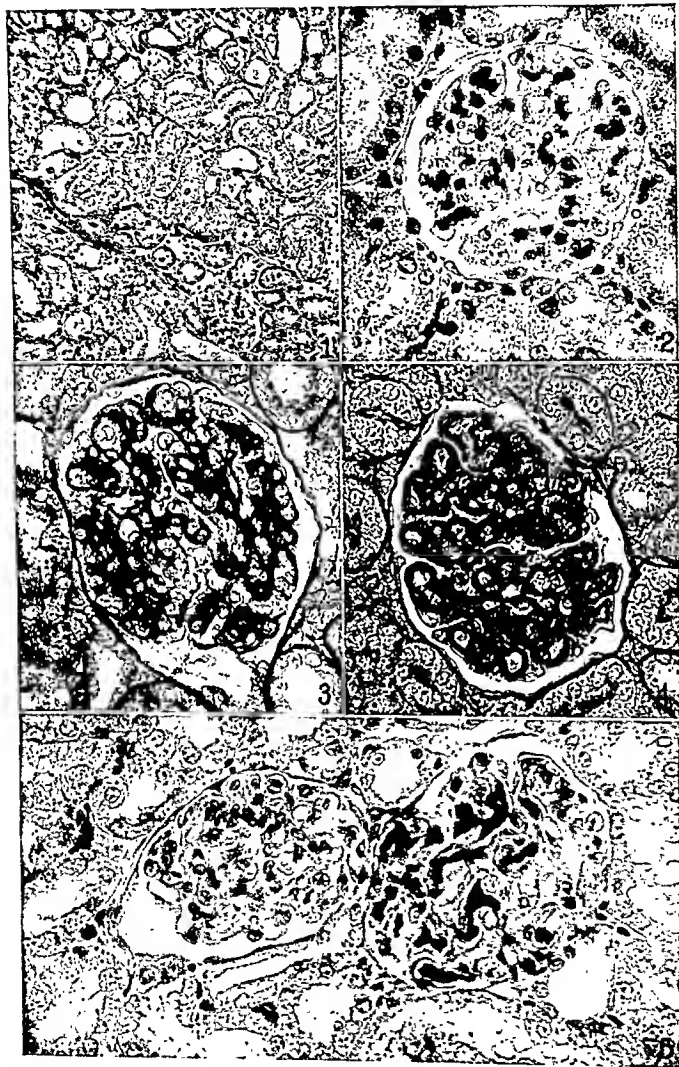
FIG. 11. Rat N-36. Heart. Scharlach R-hematoxylin stain, frozen section. The dark areas in the media of the coronary vessel represent fat. $\times 110$.

FIG. 12. Rat N-39. Received the same treatment as rat N-36, but became moribund 204 days after the last injection of anti-kidney serum. Heart. Meth-

ylene blue-eosin stain. The dark material in the muscle coat of the medium sized branch of the coronary artery is calcium. Scarring is present about the vessel and in a neighboring area of myocardium. Thickened arterioles are observed in the focus of fibrous myocarditis. $\times 195$.

FIG. 13. Rat N-39. Pancreas. Methylene blue-eosin stain. Changes in and about two small arteries are illustrated. A somewhat thickened arteriole lies below them. $\times 210$.

FIG. 14. Rat N-39. Kidney cortex. Mallory-Heidenhain stain. Connective tissue replacement is more extensive here than in Fig. 7. $\times 240$.



Photographed by Louis Schmidt

(Smadel: Nephritis induced by anti-kidney serum, III)





Photographed by Louis Schmidt

(Smadel: Nephritis induced by anti-kidney serum. IID)



Photographed by Louis Schmidt

(Smadel: Nephritis induced by anti-kidney serum III)

EXPERIMENTAL NEPHRITIS IN RATS INDUCED BY INJECTION OF ANTI-KIDNEY SERUM

IV. PREVENTION OF THE INJURIOUS EFFECTS OF NEPHROTOXIN IN VIVO BY KIDNEY EXTRACT

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The substance, present in anti-kidney serum, which induces a special type of nephritis when injected into animals, is considered by most authors to be a nephrotoxin, relatively organ specific for renal tissue. Both the literature and experiments carried out in our laboratory supporting this conception have already been presented (1). Nevertheless there are some who doubt the existence of a strictly organ specific antibody, because of cross reactions. It seemed advisable, therefore, to pursue the investigations still further; as a result the following experiments are presented which confirm the conception that nephrotoxin is quite highly, even if not strictly, organ specific.

EXPERIMENTAL

Materials and Methods

Antiserum.—Anti-rat-kidney serum was prepared by immunizing rabbits with a suspension of perfused rat kidney by a method slightly different from that previously employed (1). Suspensions of perfused rat kidney were ground without abrasive, and an effort was made, in this case, to obtain for the immunizing injections all of the finer particles of the ground kidney in addition to the extract of kidney substance in solution in the saline. The suspension was kept at a temperature of 10°C. for 24 to 48 hours while the sterility was determined. 10 cc. of the resultant suspension, which just passed through an 18 gauge needle, were injected intraperitoneally into each of six young adult male rabbits on 3 consecutive days each week. A 10 per cent concentration was employed the 1st week, 15 per cent the 2nd, and 20 per cent thereafter. Sera obtained from all of the rabbits 6 days after the twelfth immunizing treatment (4th week) contained an

appreciable amount of nephrotoxin, in most instances rather low in titer. Of the most effective serum, 0.7 cc. per 100 gm. of rat body weight was required to induce a severe nephrotoxic picture, and of the least potent, 1.4 cc. were needed. After four more treatments of the rabbits, bringing the total injections to sixteen, the nephrotoxic titer of the antisera rose, so that the most active serum, No. 4557, which was used throughout the present experiments, was capable of inducing marked nephritis when 0.5 cc., in divided doses, was given intravenously to a 100 gm. rat over a period of several days. A total amount of 0.65 cc. given in fractional doses always induced a severe nephrotoxic effect; and an occasional animal succumbed during the acute phase of the disease. It should be mentioned that this method of calculating dosages on the basis of body weight did not hold over the entire weight range for rats. Small animals (40 to 50 gm.) required more serum in proportion, and large animals (150 to 250 gm.) required relatively less than those ranging from 75 to 125 gm. Probably a calculation based on surface area would have been more accurate.

Absorbing Kidney Suspensions.—Tissue extracts for intravenous injection into rats were usually employed in 20 per cent concentrations prepared as follows: Immediately after perfusion the organ was removed aseptically, finely minced with scissors, and then ground without abrasive in a mortar. The ground tissue was weighed and diluted with sufficient saline to make a 20 per cent suspension. This was stored at 10°C. for 2 days, then centrifuged at 2,500 r.p.m. for 30 minutes. The granular sediment and floating fat were discarded, and the somewhat opalescent tan colored mid-zone was used. Extracts were also prepared from unperfused organs which had been ground and washed three times with large volumes of saline. An estimate of the protein content of tissue extracts was made by the technique of Shevky and Stafford (2).

Effect of Injecting Saline Extracts of Perfused Kidney Immediately before Administering Nephrotoxic Serum

Nephrotoxic serum acts as well in divided doses as in a single dose, and this method of administration avoids severe side effects; therefore most of the tests were carried out with this technique.

Experiment 1.—Rat N-307 (Table I), weight 50 gm., received a total amount of 0.4 cc. of nephrotoxic serum, i.e. 0.8 cc. per 100 gm. body weight, in four doses given at daily intervals. Preceding each dose the animal received 1.5 cc. of perfused kidney extract. No evidence of renal damage was detected in the following 2 weeks. The control, rat N-308, on the other hand, received the same nephrotoxic serum in similar doses preceded by normal saline and developed the usual typical evidences of nephritis.

Seven rats treated in this manner received varying amounts of perfused kidney extract and nephrotoxic serum. In four no evidence

TABLE I

Effect of Intravenous Injection of Anti-Kidney Serum Preceded by Physiological Saline

Rat N-308. Male. Received a total of 0.8 cc. per 100 gm. body weight of anti-kidney serum.

Date.	Urine examination				Body weight	Remarks	
	Albumin	Quantitative albumin	Guaiaac	Microscopic		Saline	Serum 4557
1936		per cent			gm.	cc.	cc.
Apr. 13	--		--	Normal	48	*	
" 14	--		--	"	47	1.5	0.1
" 15	--		--	"	47	1.5	0.1
" 16	--		--	"	47	1.5	0.1
" 17	+	0.8	--	No casts	50	1.5	0.1
" 18	+		--	Few granular and hyalin casts	52		
" 19	++++	3.5	--	Very many hyalin, granular, and cellular casts			
" 20	+++	1.6	--	Numerous casts	57	Slight anasarca. Experiment terminated	

Effect of Intravenous Injection of Anti-Kidney Serum Preceded by Extract of Rat Kidney

Rat N-307. Female. Received a total of 0.8 cc. per 100 gm. body weight of anti-kidney serum.

Date	Urine examination				Body weight	Remarks	
	Albumin	Quantitative albumin	Guaiaac	Microscopic		Kidney extract 20%	Serum 4557
1936		per cent			gm.	cc.	cc.
Apr. 13	Faint trace	0.03	--	Normal	50	*	
" 14	--		--	"	50	1.5	0.1
" 15	Faint trace		--	"	47	1.5	0.1
" 16	--		--	"	49	1.5	0.1
" 17	--		--	"	50	1.5	0.1
" 18	Faint trace		--	"	51		
" 19	" "		--	"	50		
" 20	" "	0.06	--	"	51		
" 22	Trace	0.4	--	"	55		
" 24	Faint trace		--	"	58		
" 27	--		--	"	62	Animal healthy. Experiment terminated	

* Control period of observation.

TABLE II

Effect of Intravenous Injection of Anti-Kidney Serum Preceded by Extract of Rat Liver

Rat N-323. Female. Received a total of 0.6 cc. per 100 gm. body weight of anti-kidney serum.

Date	Urine examination				Body weight	Remarks	
	Albumin	Quantitative albumin	Guaiae	Microscopic		Liver extract 20%*	Serum 4557
1936		per cent			gm.	cc.	cc.
May 18	—		—	Normal	63	†	
" 19	—		—	"	64	1.5	None
" 20	—		—	"		1.5	0.1
" 21	Faint trace		—	"		1.5	0.1
" 22	" "		—	"		1.5	0.1
" 23	Heavy trace	0.5	—	"	62	1.5	0.1
" 24	++		—	Occasional hyalin cast			
" 25	+++	1.8	—	Few granular and hyalin casts			
" 26	++++	2.7	—	Few granular and cellular casts	74	Slight anasarca	
" 27	++++	3.2	—	Many casts	86	Marked anasarca, Experiment terminated	

Effect of Intravenous Injection of Anti-Kidney Serum Preceded by Extract of Rat Kidney

Rat N-326. Female. Received a total of 0.6 cc. per 100 gm. body weight of anti-kidney serum.

Date	Urine examination				Body weight	Remarks	
	Albumin	Quantitative albumin	Guaiae	Microscopic		Kidney extract 20%†	Serum 4557
1936		per cent			gm.	cc.	cc.
May 23	Faint trace	0.04	—	Normal	68	†	
" 24	—		—	"	69	2.0	0.1
" 25	Faint trace		—	"	70	2.0	0.1
" 26	Trace	0.2	—	Rare cast	67	2.0	0.1
" 27	"	0.1	—	No casts		2.0	0.1
" 28	Faint trace	0.02	—	" "	71		
" 29	" "	0.08	—	Rare cast	69		
June 1	" "	0.05	—	No casts	68		
" 6	Trace	0.1	—	" "	77		
" 12	Faint trace	0.05	—	" "	84	Animal healthy. Experiment terminated	

* Liver extract contained 0.43 per cent protein.

† Control period of observation.

‡ Kidney extract contained 0.28 per cent protein.

of renal damage was obtained, although the amounts of the serum given were sufficient to induce severe nephritis in controls. These four rats received 1.5 or 2.0 cc. of 20 per cent kidney extract followed by 0.1 cc. of serum 4557 on 4 consecutive days. No histopathological lesions were found in any of the animals that showed clinically an inhibition of the nephrotoxic action of anti-kidney serum.

When, on the other hand, the amount of kidney extract was reduced below a certain minimum, the inhibitory effect was less apparent.

For example, a rat weighing 59 gm. received four daily treatments consisting of 1.0 cc. of 40 per cent kidney extract followed immediately by 0.15 cc. (0.25 cc. per 100 gm. body weight), of serum 4557. The nephrotoxin was only partially inhibited. Another animal of the same weight as N-326 (Table II) was treated with identical amounts of the same kidney extract and serum and developed severe nephritis. In this instance the kidney extract was filtered through a Seitz pad which changed it from an opalescent solution to a clear one and probably reduced its protein content, although no quantitative determination was made. The final animal in this group received a total of 2.0 cc. of 20 per cent kidney extract and 0.6 cc. of anti-kidney serum per 100 gm. of body weight but half of the extract was given in divided doses over a period of 3 consecutive days before the serum injections were begun. The first 0.2 cc. of serum followed the administration of kidney extract by 24 hours and the two subsequent serum injections were preceded by 0.3 cc. and 0.7 cc. of extract respectively. The failure of this animal to show inhibition of nephrotoxin suggests that adequate amounts of kidney extract must be given very shortly before the anti-kidney serum in order to prevent nephritis.

Effect of Injecting Saline Extracts of Perfused Liver Immediately before Administering Nephrotoxic Serum

It was obviously necessary to learn whether this inhibitory effect, demonstrable *in vivo*, was organ specific, especially in view of previous experiments (1) in which it was shown that liver extracts in relatively large amounts apparently neutralized the nephrotoxic effect when mixed with nephrotoxic serum *in vitro* before the latter was given to rats.

Experiment 2.—Rat N-323, weighing 64 gm., received an initial intravenous injection of 1.5 cc. of 20 per cent perfused liver extract which contained 0.43 per cent of protein. Slight cutaneous flushing and moderately increased respiratory rate were the only effects noted. On each of the following 4 days the same amount

TABLE II

Effect of Intravenous Injection of Anti-Kidney Serum Preceded by Extract of Rat Liver

Rat N-323. Female. Received a total of 0.6 cc. per 100 gm. body weight of anti-kidney serum.

Date	Urine examination				Body weight	Remarks	
	Albumin	Quantitative albumin	Guaiac	Microscopic		Liver extract 20%*	Serum 4557
1936		per cent			gm.	cc.	cc.
May 18	—		—	Normal	63	†	
" 19	—		—	"	64	1.5	None
" 20	—		—	"		1.5	0.1
" 21	Faint trace		—	"		1.5	0.1
" 22	" "		—	"		1.5	0.1
" 23	Heavy trace	0.5	—	"	62	1.5	0.1
" 24	++		—	Occasional hyalin cast			
" 25	+++	1.8	—	Few granular and hyalin casts			
" 26	++++	2.7	—	Few granular and cellular casts	74	Slight anasarca	
" 27	++++	3.2	—	Many casts	86	Marked anasarca. Experiment terminated	

Effect of Intravenous Injection of Anti-Kidney Serum Preceded by Extract of Rat Kidney

Rat N-326. Female. Received a total of 0.6 cc. per 100 gm. body weight of anti-kidney serum.

Date	Urine examination				Body weight	Remarks	
	Albumin	Quantitative albumin	Guaiac	Microscopic		Kidney extract 20%†	Serum 4557
1936		per cent			gm.	cc.	cc.
May 23	Faint trace	0.04	—	Normal	68	†	
" 24	—		—	"	69	2.0	0.1
" 25	Faint trace		—	"	70	2.0	0.1
" 26	Trace	0.2	—	Rare cast	67	2.0	0.1
" 27	"	0.1	—	No casts		2.0	0.1
" 28	Faint trace	0.02	—	" "	71		
" 29	" "	0.08	—	Rare cast	69		
June 1	" "	0.05	—	No casts	68		
" 6	Trace	0.1	—	" "	77		
" 12	Faint trace	0.05	—	" "	84	Animal healthy. Experiment terminated	

* Liver extract contained 0.43 per cent protein.

† Control period of observation.

‡ Kidney extract contained 0.28 per cent protein.

of liver extract was given, followed by 0.1 cc. of anti-kidney serum; hence a total of 0.62 cc. per 100 gm. body weight of this particular nephrotoxic serum was injected. The animal developed typical nephritis clinically; and the kidneys removed 3 days after the last treatment revealed both macro- and microscopic evidence (3) of this type of nephritis. A control rat, N-326, that received the same serum, preceded by kidney instead of liver extract, remained healthy.

This, and similar experiments, indicate that the nephrotoxic principle in anti-kidney serum is specifically absorbed by extract of renal substance.

*Effect of Injecting Saline Immediately before Administering
Nephrotoxic Serum*

The question naturally arises as to whether a dilution factor might have been responsible, in part, for the less marked effect of nephrotoxic serum given after injecting kidney or other extracts. This apparently can be answered in the negative, for repeatedly it has been shown that anti-kidney serum was as effective when diluted *in vitro* as when given in a concentrated form. The marked nephritis found in control rat N-308 (Table I), also demonstrates that dilution of the blood with saline previous to the administration of anti-kidney serum does not diminish its nephrotoxic influence.

Effect of Saline Extracts of Unperfused Kidney and Liver

Attempts were made to repeat the foregoing experiments with saline extracts of kidneys and livers which were removed from rats immediately after they were exsanguinated. The organs were finely minced and ground in mortars, then washed with large amounts of physiological salt solution prior to extraction. Severe general shock-like reactions usually followed the intravenous injection of appreciable amounts of these extracts, whether they were given alone or followed by nephrotoxic serum. This experience convinced us that for the demonstration of antinephrotoxic effects of organ extracts *in vivo* a suitable preparation of the solutions or suspensions is necessary.

DISCUSSION

The present experiments have shown that adequate amounts of a saline extract of rat kidney, when injected intravenously in rats

immediately prior to the administration of anti-rat-kidney serum, inhibit the nephrotoxic action of the serum. On the other hand, a preparatory treatment with rat liver extract which absorbs the nephrotoxin *in vitro*, or with saline, does not inhibit the nephrotoxic action of anti-kidney serum.

The ability of kidney extract to neutralize *in vivo* the nephrotoxic action of anti-kidney serum presumably depends on a combination of nephrotoxin with the soluble kidney material either in the blood stream or before the antibody affects the cells of the recipient kidney. The antigen-antibody combination within the body is, under these circumstances, not sufficient in itself to induce the nephrotoxic picture nor is any secondary product evolved which induces the condition. This is not an isolated example of an antigen-antibody combination being harmless *in vivo*; for it has been shown (1, 4) that anti-rat serum precipitins, prepared by immunizing rabbits with rat serum, can be given intravenously to rats in large amounts without inducing untoward signs or pathological lesions. To attain this effect the animal must be prepared by several daily increasing doses of anti-rat serum precipitins, beginning with an amount too small to induce a notable effect. Renal lesions may result from simple reverse anaphylaxis in rats (1, 4) but they are distinctly different from those observed after treatment with nephrotoxin. Masugi's (5) observations on the kidneys of sensitized rabbits that received shocking doses of antigen by various routes led him to conclude that any *in vivo* antigen-antibody reaction might result in a glomerulonephritis comparable to human nephritis. Our experience does not support such a conclusion.

Although the present observations indicate a strict organ specificity of nephrotoxin for kidney tissue, still the concept of relative organ specificity previously presented cannot be entirely discarded. The basis for considering nephrotoxin only relatively specific was twofold (1): first, while kidney tissue extract readily absorbed nephrotoxin *in vitro*, liver tissue extract, used repeatedly in large amounts, also removed it; and, second, a weak nephrotoxic effect was occasionally demonstrated in antisera prepared against organs other than kidney, *viz.* brain (1) and liver (6). Even though the absorption with liver might be interpreted as indicating a species specific absorption and

not an overlapping organ specific absorption, because large amounts of liver tissue were required, still the second point must be remembered in interpreting the present experiments. The entire body of evidence so far presented, however, points to a relatively strict organ specificity of nephrotoxin in properly prepared anti-rat-kidney serum.

SUMMARY

A saline extract of perfused rat kidney administered intravenously to rats immediately before injecting an anti-rat-kidney serum, by the same route, prevents renal damage. A preliminary injection of physiological salt solution or of an extract of perfused rat liver has no preventive effect.

These findings are a further indication that the nephrotoxic effect induced by anti-kidney serum is dependent upon a relatively organ specific antibody, nephrotoxin.

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AN IMPROVED AIR-DRIVEN TYPE OF ULTRACENTRIFUGE FOR MOLECULAR SEDIMENTATION

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INTRODUCTION

In 1925 Henriot and Huguenard (1) described a new method for obtaining high rotational speeds without the use of mechanical bearings. Employing a whirling layer of air issuing under pressure from properly directed jets for both supporting and driving a small cone-shaped rotor measuring 11.7 mm. in diameter, they reported attaining speeds up to 660,000 revolutions per minute. In another paper (2), 2 years later the same authors described their attempts to adapt the air-drive principle for several practical purposes. These included a Sharples type of centrifuge with a cylinder weighing 2,500 gm., which they reported to have successfully spun at a speed of 65,000 R.P.M. They also claimed to have attained speeds up to 360,000 R.P.M. with a rotor carrying a small mirror. The new method for obtaining high rotational speeds without the use of mechanical bearings was soon confirmed by Beams (3). In order to eliminate some of the rather objectionable vibration, Beams introduced certain modifications into the design of Henriot and Huguenard and obtained rotational speeds up to 180,000 R.P.M. with a rotor measuring about 30 mm. in diameter and carrying a small stellite mirror. He was also successful in photographing electric sparks as reflected from the rapidly rotating mirror (4). Substituting compressed hydrogen in place of air for driving purposes, and using a plain rotor only 9 mm. in diameter, Beams, Pickels, and Weed (5) reported attaining rotational speeds of over 1,200,000 R.P.M.

The possibility that this method of obtaining high rotational speeds might be utilized with advantage in designing and constructing high speed centrifuges suitable for chemical and biological investigations naturally suggested itself, and subsequently there have appeared several reports of such attempted applications. The rapidly spinning rotor has been employed as a centrifuge in studying the effects of high centrifugal forces upon certain cells by Harvey (6, 7), by Beams and King (8, 9), and by Guyer and Claus (10). McIntosh (11) reported using it for the centrifugation of bacteriophage and fowl sarcoma virus. Beams, Weed, and Pickels (12, 5) have described arrangements designed for the separation of protein molecules and other small particles in solution. McBain and O'Sullivan (13, 14)

have given detailed descriptions of an apparatus involving the use of a small air-driven rotor for the determination of the sedimentation constants of protein molecules in solution. These authors claimed to have been successful in recording photographically the sedimentation boundaries of certain large monodisperse proteins.

A considerable amount of research has been carried out by the writers in an effort to utilize the air-drive principle of Henriot and Huguenard in the design and construction of a high speed centrifuge suitable for the study of filterable viruses. For this purpose, two types of centrifuges seemed essential: one, for the concentration and purification of viruses, and therefore capable of accommodating a relatively large amount of fluid; the other, with a suitable optical system, for the study of the physical characteristics of the viruses by determining their sedimentation constants in an intense centrifugal field. In the course of these investigations, it soon became evident that high rotational speeds were obtainable by this method only with relatively small rotors, the dimensions of which were wholly unsuitable for our purpose. A number of models were built, and rotors varying from 50 to 200 mm. in diameter were tried. With the increase of the rotor diameter and the resulting increase in the surface area of the rotor exposed to air friction, there was a corresponding decrease in the maximum speed obtainable. A speed of 16,000 R.P.M. could not be exceeded with a rotor measuring 200 mm. in diameter, regardless of the large volume of the compressed air under high pressure employed for driving purposes (Bauer and Pickels (15)). Moreover, we were not able to devise a satisfactory arrangement to prevent the development of temperature gradients and the resulting convection currents which interfere with normal sedimentation. These limitations, coupled with certain other difficulties encountered in these investigations, led us to abandon the hope of adapting the simple air turbine of Henriot and Huguenard as a high speed centrifuge suitable for the studies in which we were especially interested.

Pickels and Beams (16) were the first to describe a new type of air turbine drive with which it was possible to obtain high rotational speeds with larger rotors without exposing them to appreciable air friction. To a friction clutch, inserted into a small cone-shaped air-driven rotor of the Henriot and Huguenard type, they attached a length of straightened piano wire which was extended downward through an oil gland into a vacuum chamber and there fastened to a larger rotor. This wire served as a drive shaft. At the lower end of the rotor a stabilizing device was used. When a high degree of vacuum was maintained in the chamber housing the larger rotor, only a relatively small amount of driving energy was necessary to attain rotational speeds which were limited only by the strength of the material used for the rotor. Later this driving mechanism was somewhat modified by Beams and Pickels (17), in that the friction clutch and the stabilizer were eliminated, a certain degree of flexibility was given to the mounting of the oil gland, and two separate air systems were used, one for supporting and the other for driving the turbine. Utilizing the principles described by Pickels and Beams (16), Biscoe, Pickels, and Wyckoff (18) constructed a centrifuge in which they used a

friction clutch in the turbine as well as two air systems for supporting and driving purposes. Large circular duralumin rotors measuring about 176 mm. in diameter and carrying transparent cells similar to those designed by Svedberg and his coworkers (19) were used. They also adapted the optical systems of Svedberg and were able to record photographically the sedimentation of protein molecules in their centrifuge.

Recently Bauer and Pickels (20) described a high speed vacuum centrifuge designed for the concentration and purification of filterable viruses. An air turbine drive, which was a further modification of those described by the investigators referred to above, proved to be highly efficient for this centrifuge. The turbine and its load were supported by a new type of air-bearing arrangement in which was employed for the first time a special floating unit designed to absorb more efficiently vibrations generated in the turbine and to give it an increased stability at all speeds. This bearing unit was made to function entirely independently of the driving system. The new type of driving mechanism was provided with a set of reversed air jets by which the rotor could be rapidly decelerated and within a short time brought to rest from high speed. With this type of air bearing, only a comparatively small amount of compressed air was required to spin large and heavy rotors at speeds which were limited solely by the strength of the material from which the rotors were made. The application of this driving mechanism to a molecular centrifuge for the determination of the sedimentation constants of proteins has now proved equally successful. The rotation of an oval-shaped rotor at very high speed has been steady and vibrationless, and there has been no evidence of convection currents interfering with the sedimentation of the protein molecules. Following is a detailed description of this centrifuge assembly, including the adaptation of Svedberg's optical system.

The Improved Centrifuge

Driving Mechanism.—The driving mechanism has already been fully described in a recent report (20), and in the present paper only certain features are briefly mentioned in connection with its adaptation to the molecular ultracentrifuge. The one major change introduced is a reduction of the diameter of the driving turbine to 35 mm. in order to minimize air friction, inasmuch as the rotors of the molecular ultracentrifuge are lighter than those described in the previous report

and are run at higher speeds. In Figs. 1 and 2 are shown the arrangement used in the mounting of the mechanism on the vacuum chamber and the details of the system used for collecting the lubricating oil entering the chamber. In order to provide the necessary degree of flexibility and dampening, the whole driving mechanism is mounted on a fairly heavy metal plate (1, Fig. 1) which rests on four small rubber cushions (2, Fig. 1). The vacuum seal between the chamber and the driving mechanism is effected by means of soft, thick walled rubber tubing (3, Fig. 1) which connects the lower stem of the oil gland (4, Fig. 1) to a sleeve (5, Fig. 1) fastened to the top plate of the vacuum chamber. Lubricating oil of a low vapor tension, forced under continuous pressure into the central cavity of the oil gland (6, Fig. 1), lubricates the bearings and also serves to form a vacuum seal about the drive shaft. Oil escaping from the upper bearing is drained into a container located above the chamber. For collecting oil that escapes from the lower bearing into the vacuum chamber, a duralumin disc with a diameter slightly larger than the aperture in the circular oil collector which surrounds it (7, Fig. 1), is fitted tightly on the drive shaft beneath the lower bearing. Oil thrown from the disc into the collector is drained through a copper tubing into a cup fastened to the top of the chamber (8, Fig. 1). The oil collected in the chamber during a run has averaged about 1 cc. per hour, and consequently its removal is required only at infrequent intervals.

Vacuum Chamber.—The vacuum chamber in which the centrifuge rotates consists of a cylinder and two end plates as shown in Figs. 1 and 2. The cylinder (9, Figs. 1, 2) is of chrome nickel steel alloy, S.A.E. 3140, and its dimensions are: height 28 cm., inside diameter 22.8 cm., and wall thickness 7.6 cm. After casting, it is machined to the desired shape and heat-treated to give a maximum tensile strength to the steel. After the heat treatment the ends are ground smooth and parallel to facilitate securing satisfactory vacuum seals when joined with the end plates. The end plates are made of hot rolled steel; they measure 52.5 cm. square and are machined smooth and flat. Through the plates at each corner there is drilled a 5 cm. hole. The thickness of the top plate (10, Figs. 1, 2) is 3.8 cm., and that of the bottom plate (11, Figs. 1, 2) is 5 cm. The chamber rests on four steel legs (12, Fig. 2) which are also fashioned into the form of bolts. The diameter of each leg is 7.5 cm. to a height of 30.5 cm., and from there on is reduced to 5 cm. The upper ends are threaded and provided with heavy steel nuts (13, Figs. 1, 2). Passing through the holes in the corners of the bottom plate and thus bringing the latter to rest on the shoulders, the bolts extend along the outer wall of the cylinder and then pass through the holes at the corners of the top plate with a slight clearance. This arrangement provides for an automatic alignment of the optical light path when the chamber is closed, and also permits the end plates to be securely bolted together when the chamber is closed during a run. Thin rubber washers (14, Fig. 1) and lubriscal are used to effect a vacuum-tight seal between the cylinder and the end plates. Into the edge of the bottom plate there are drilled two holes which have openings into the chamber itself. One of these leads to the vacuum pump, and the other to the vacuum gauge. In addition there are drilled

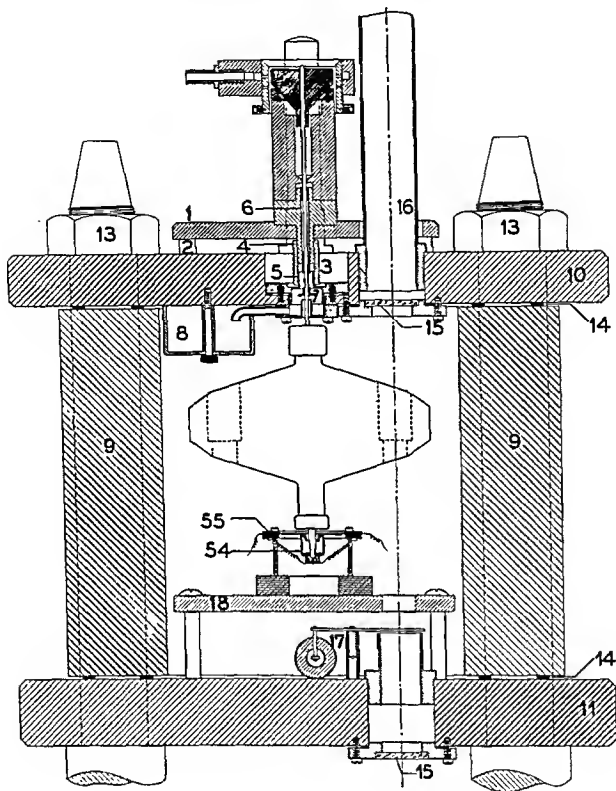


FIG. 1. Vertical cross-section view of vacuum chamber and driving mechanism.

through the thickness of the bottom plate several smaller holes which are tightly closed with rubber stoppers. Wires passing through these stoppers serve as electrical leads for an electromagnetic shutter and for the temperature-measuring devices.

either in an opened or a closed position for any length of time without maintaining a continuous supply of electrical energy which might result in an undesirable amount of beating in the chamber. The shutter has the additional purpose, especially during the opening and closing of the vacuum chamber, of protecting the bottom window from dust, oil droplets, etc.

The bottom of the vacuum chamber is provided with an elevated flooring (18, Figs. 1, 2) which is made of brass sheeting 13 mm. thick and mounted on three legs above the shutter. The purpose of this arrangement is twofold: it serves as a protection to the shutter, the bottom window, and the electrical wiring system in the unexpected event of the rotor's dropping from the drive shaft; and it also furnishes a convenient support for the temperature-measuring device described below.

Rotor.—The work of Svedberg and his associates as well as our own experience has clearly demonstrated that ultracentrifuge rotors with transparent cells can be made to withstand considerably greater centrifugal force if they are made oval rather than circular in shape. The two holes cut into the rotor opposite each other to accommodate the transparent cell and its counterbalance result in an asymmetrical distribution of stresses during rotation. The danger of rupture is much diminished and the maximum speed obtainable materially increased if the excess of metal is removed from the solid sections along the periphery farthest from the holes. Strength is also added to the rotor if its thickness is progressively increased from the periphery toward the center as much as the design permits.

Among the various metals suitable for rotors, an aluminum alloy known as 14 ST has proved most satisfactory for our purposes (21). It has the chief advantage over special steel alloys in that the stock supplied by the manufacturers, although already heat-treated to bring out its maximum tensile strength, is relatively soft and very easily machined, and the rotor can be finished completely without subjecting it to the additional heat treatment and subsequent grinding process that are necessary with steel alloys. It is also sufficiently malleable to allow a slight flow under tension, a property which minimizes the danger of generating localized stresses and strains that are apt to initiate a rupture at high speed. Moreover, having only about one-third the density of steel and being very much softer than a heat-treated steel alloy, a duralumin rotor exploding at excessive speed is not nearly so dangerous as a steel one. According to the stress-strain data¹ available, the 14 ST alloy has an ultimate tensile strength of 67,400 pounds per square inch, yield strength of 57,000 pounds per square inch, elongation 15.8 per cent, reduction of area 31.1 per cent, Brinell hardness 131.5, and modulus of elasticity 10,400,000 lb. per sq. in. The specific tensile strength of this alloy com-

¹ We are indebted for these data to Mr. R. L. Templin, Chief Engineer of Tests, Aluminum Research Laboratories of the Aluminum Company of America, New Kensington, Pennsylvania.

pares favorably with that of a steel alloy having a tensile strength of 200,000 lb. per sq. in., *viz.*

$$\text{steel: } \frac{\text{tensile strength}}{\text{density}} = \frac{200,000}{7.8} = 25,604; \text{ 14 ST: } \frac{67,400}{2.8} = 24,070$$

The rotor as shown in Figs. 2, 3, 4, and 5 is made on an ordinary lathe. No special precision equipment is needed and no further balancing has been found to be necessary after the machining. It is machined from a solid piece of forged alloy which is x-rayed to ensure the absence of flaws. In the first machining operation the rotor is made circular, with a 10 cm. flange located 1 cm. above the bottom tip. The two cell holes located exactly opposite each other are bored on a milling machine. The approximately oval shape is given to the rotor in the

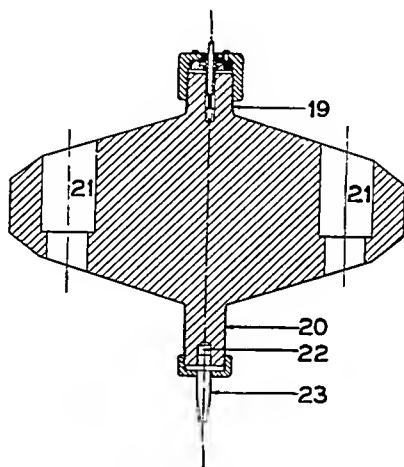


FIG. 3. Vertical cross-sectional view of rotor at its greatest diameter.

following manner: The bottom stem, 1.9 cm. in diameter and extending 1 cm. below the flange, is fitted very closely into a hole bored through a 1.4 cm. steel plate, which is then bolted firmly to the lathe's face plate in such a position that the hole is 3.2 cm. from the axis of the lathe spindle. Six short bolts extending through the flange and threaded into the steel plate serve to hold the rotor rigidly against the steel plate as it revolves in the lathe, which is adjusted to cut the shape indicated. The exact operation is then duplicated on the other half of the rotor by turning it 180° and rebolting it against the steel plate. The final step is to place the rotor between lathe centers for cutting away the flange and for threading the two stems.

The periphery of the rotor when finished has a maximum diameter of 185 mm. (Fig. 3) and a minimum diameter of 170 mm. (Fig. 4). At the top there is a threaded stem 22 mm. in diameter and 19 mm. in length (19, Figs. 3, 4), and at the

bottom is another, 19 mm. in diameter and 25 mm. in length (20, Figs. 3, 4). The over-all height of the rotor, including the two stems, is 135 mm. From these the thickness tapers down to 25 mm. at the periphery nearest to the cell holes, and to 2 mm. in the plane of the shortest diameter. The center of each cell hole is at a distance of 65 mm. from the axis of rotation. These holes (21, Figs. 3, 5) have a diameter of 25 mm. for about five-sevenths of their depth, the remainder being reduced to 19 mm. The shoulders in the holes serve as stops to ensure bringing the cell and a metal plug used for counterbalancing into dynamically balanced positions. They also eliminate the possibility of having any light pass between the cell and the rotor. In the lower stem of the rotor there is a 6 mm. hole (22, Figs. 3, 4) which serves to center a smaller steel stem (23, Figs. 3, 4) that is held in position by means of a screw cap. This stem, 6 mm. in diameter at the top and

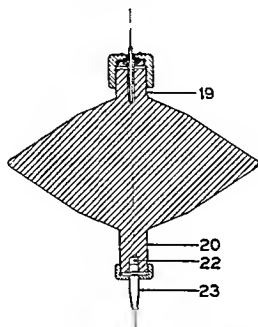


FIG. 4. Vertical cross-sectional view of rotor at its shortest diameter.

tapered at its lower end, extends 22 mm. below the screw cap and forms a part of the temperature-measuring device.

The rotor is fastened to the drive shaft with a special chuck, the details of which have already been described (20). When assembled complete with chuck, cell, its counterbalance, etc., the rotor weighs 3,430 gm. A considerable number of runs, each from 2 to 3 hours' duration, have been carried out with this rotor at a speed of 60,000 R.P.M., and although measured frequently with micrometer calipers, it has shown no evidence of permanent stretching or deformation.

Cells.—The cell, designed to accommodate a sector-shaped column of fluid, is constructed along the general lines described by Svedberg (19). In Fig. 6 is shown a cross-sectional view of the cell when assembled, and in Fig. 7 its various parts are shown separately. A duralumin cylinder (24, Figs. 6, 7), which measures 25 mm. both in diameter and in height, serves as the outside casing of the cell. The inside diameter of this casing is 22 mm. for 19 mm. of its depth and, in order

to provide a supporting shoulder, the diameter is reduced to 16 mm. for the remainder. At this end of the casing there are two holes drilled in its wall to accommodate the two pins of a special wrench used in assembling the cell. The center piece (26, Figs. 6, 7) with its sectoral opening is made of pontalite² and has an outside diameter of 22 mm. At both ends there are machined out cavities, each to the depth of 4.75 mm., leaving rims with a wall thickness of 1.5 mm., and a flat

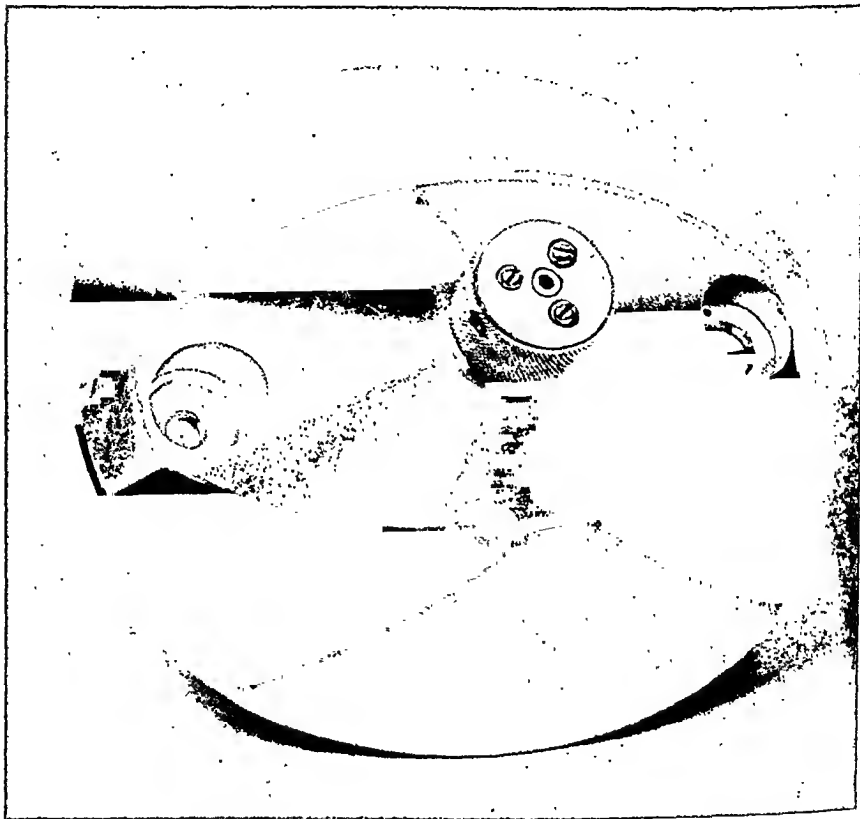


FIG. 5. Photograph showing actual appearance of rotor.

central section (27, Fig. 6) 3 mm. thick. A sector-shaped opening, having an angular width of 4° and a length of 15 mm. (28, Figs. 6, 7) is cut into this central section. Narrow, circular, concentric grooves, each 0.04 mm. deep, are machined into either face of the central section. This corrugation extends from the ends of the sectoral aperture to the junctions of the projecting rims. In assembling

² A new clear, plastic material manufactured by E. I. Du Pont De Nemours and Company.

the cell, a wet pliofilm³ washer, 0.06 mm. thick (25, Fig. 7), is placed against each of these corrugated sections, and crystal quartz discs measuring 19 mm. in diameter and 5 mm. thick (29, Figs. 6, 7) are then placed on the washers, forming an enclosed sector-shaped cavity in the center piece. This assembled section of the cell is then inserted into the casing and brought to rest on its shoulder (30, Fig. 6) which is provided with a paper washer (31, Fig. 7) to protect the lower quartz disc. A similar washer placed upon the upper disc is followed by a pressure ring (32, Figs. 6, 7) and the whole assembly clamped together with a screw ring (33,

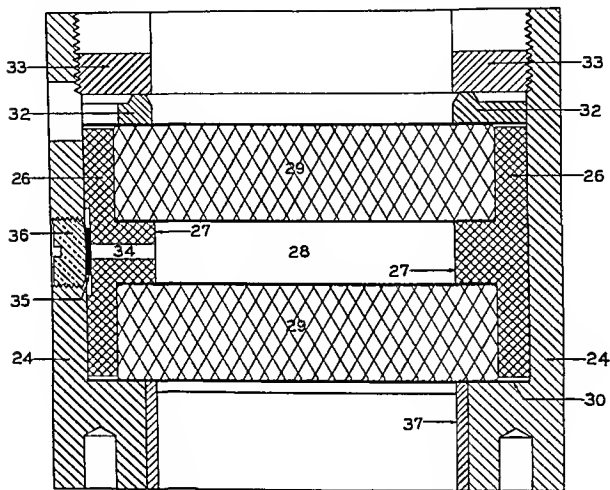


FIG. 6. Vertical cross-sectional view of cell, showing details of construction.

Figs. 6, 7). The fluid is introduced into the sectoral cavity of the cell through a small hole (34, Fig. 6) drilled through the wall of the center piece; evaporation is prevented by an air-tight seal effected with a small rubber disc (35, Figs. 6, 7) and a screw plug (36, Figs. 6, 7). A cup-shaped light sector (37, Figs. 6, 7) with a sectoral opening of 3° angular width and a length of 15 mm. is inserted into the

³ A clear, somewhat elastic sheeting resembling cellophane in appearance, manufactured by the Goodyear Rubber Company.

lower end of the cell casing. Instruments constructed especially for the purpose are used for tightening the screw ring and aligning the sector with the center piece.

Cells constructed in the manner described above have been used repeatedly for centrifugation at a speed of 60,000 R.P.M., and although the hydrostatic pressure exerted by the fluid column at this speed exceeds 300 atmospheres, they have shown no evidence of leakage. On the other hand, fracture of a quartz disc has occurred occasionally.

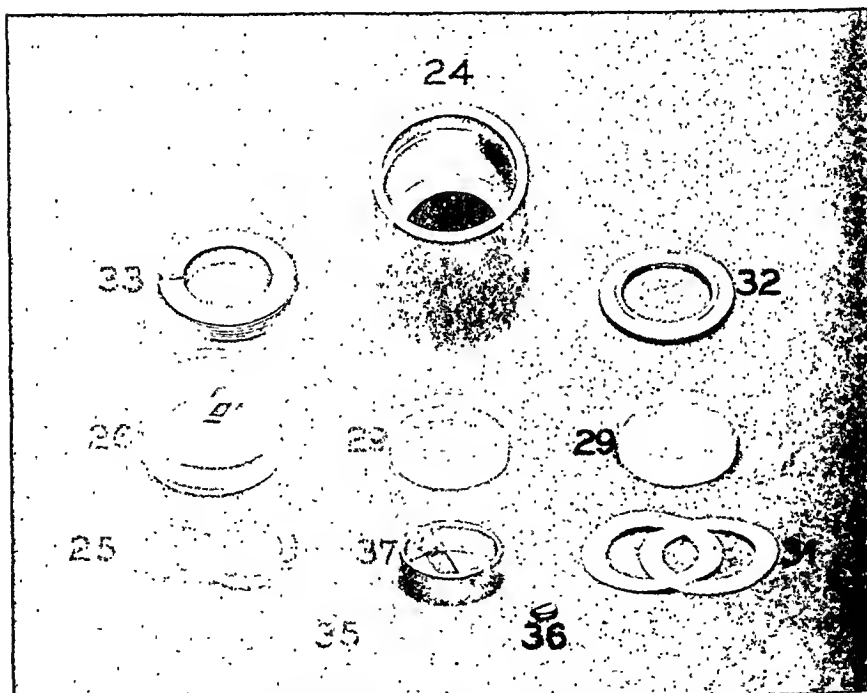


FIG. 7. Disassembled cell, showing parts separately.

Optical System.—With certain minor modifications, the optical systems adapted to the air-driven ultracentrifuge for the photographic recording of molecular sedimentations are similar to those designed by Svedberg for his ultracentrifuge with the oil turbine drive. The essential elements comprising the major portions of these systems are shown schematically in Fig. 8. Photographs in Figs. 9 and 10 show some of the features in detail. The most important items in these systems are the two photographic lenses.⁴ One of these (38, Fig. 8) used for the so called absorption method, is a quartz-fluorite achromatic triplet combination. Having a focal length of 100 cm. and an aperture f. 36, it is made especially for ultraviolet

⁴ Made by Adam Hilger, Ltd., London.

photography. The other (39, Fig. 8), employed for the refractive index method, is a quartz-glass triplet corrected for 3,500 to 4,500 Å and having a focal length of 150 cm.

As seen from the photographs in Figs. 9 and 10, the entire centrifuge assembly occupies two rooms. Heavy seamless steel pipes (40, 41, Figs. 9, 10), which extend through the wall between the rooms, form the main body of the cameras. In the room which houses the centrifuge (Fig. 9) the ends of these camera tubes are mounted one above the other in a heavy steel support, which in turn is bolted to a large channel-shaped concrete block. The upper end of each tube is mounted on a separate concrete block. The lower end of each tube is mounted on a square concrete block. In order to obviate the transmission of troublesome vibration from the building to the camera systems,

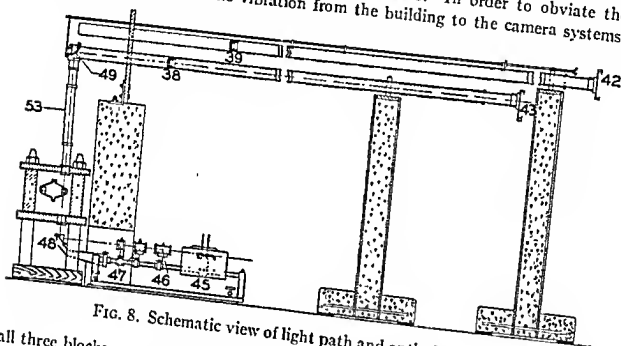


Fig. 8. Schematic view of light path and optical system.

all three blocks as well as the centrifuge chamber itself are mounted on soft rubber pads.

Inside each steel camera tube there is a smooth fitting brass tube having a length of 300 cm. Each of these inner tubes has its camera lens fitted at one end and a photographic plate holder (42, 43, Figs. 8, 10) at the other end. Correct adjustment for the necessary focusing is permitted by the telescopic action of this double tube arrangement.

Through the lower middle section of the concrete block adjacent to the centrifuge is an opening 61 cm. wide and 38 cm. high. Into this extends the optical bench (44, Fig. 9) on which can be mounted either a projection system for index of refraction measurements or an illuminating system for absorption studies. The latter system, arranged for work in the middle ultraviolet, is illustrated in the drawing (Fig. 8). The light which eventually forms the photographic image is furnished by a 220 volt mercury arc lamp (45, Figs. 8, 9). It passes successively through the water filter (46, Fig. 8), the chlorine, and the bromine gas filters

(47, Fig. 8). The approximately monochromatic ultraviolet light transmitted is then directed with a crystal quartz prism (48, Fig. 8) through the centrifuge into the camera head, and from there by means of a sputtered aluminum mirror (49, Fig. 8) to the camera lens. The sputtered mirror is mounted at 45° on a base which is held by springs against the pointed tips of three screws. Movement to two of these screws can be transmitted from either of two sets of adjusting wheels located near the photographic plate holders. The motion is transferred through the long steel rods (50, Figs. 9, 10) and the rubber belting (51, Fig. 9) to the two pulley wheels (52, Fig. 9) connected to the screws. The arrangement affords a simple means for slightly tilting the mirror in any direction and centering the photographic image at the plate holder. The camera head, which connects with the centrifuge chamber through a flexible cloth bellows (53, Figs. 8, 9), is made interchangeable in order that it may be fitted to the upper camera tube when index of refraction studies are being made.

Vacuum System.—Since the amount of frictional heat developed in the rotor is dependent on the degree of vacuum obtained in the centrifuge chamber, it is important that the vacuum system have a maximum of efficiency. A Cenco megavac pump is used to maintain a high degree of vacuum in the chamber during a run, and a McLeod gauge is used to measure the pressures. In the vacuum line leading to the gauge there is a three-way valve made of glass and fitted with mercury seals. One setting of the valve connects the gauge with the centrifuge chamber, and another setting opens the system to the atmosphere and allows the chamber to be filled with air. Metal tubing is used for the vacuum lines as far as possible, and rubber tubing is used only for connections which must be flexible.

Temperature Measurements.—For a quantitative determination of molecular sedimentation in a centrifugal field, it is essential that the temperature of the material be known at all times during centrifugation. It is also desirable to keep a record of temperature fluctuations in other parts of the centrifuge. For such temperature measurements, copper-constantan thermocouple junctions are connected to the oil bearings, to the chamber wall, and to a small cup of mercury into which projects the lower steel stem of the rotor. This cup shown in Figs. 1 and 2, is part of a simple device which has been found capable of registering the temperature of a rotor at high speed to within a few tenths of a degree Centigrade, even when the rotor and the surrounding chamber have a temperature difference of several degrees.

The major portion of the cup (54, Fig. 1) is machined of hard rubber, the walls being made as thin as possible to ensure a low thermal capacity. The central cavity has a minimum diameter of 7 mm. at the tapered entrance, a diameter of 16 mm. for the next 10 mm. of its depth, and a diameter of 7 mm. for the lowest section, which is 6 mm. deep. It is this section which is filled with mercury and has fitted over it a thin steel cap connected to the thermocouple junction. In order to insulate the cup thermally as well as possible, it is rigidly supported by a series of silk threads which connect to a bakelite ring (55, Fig. 1). The thermocouple leads to the cup are of fine wire, coiled to reduce heat losses.

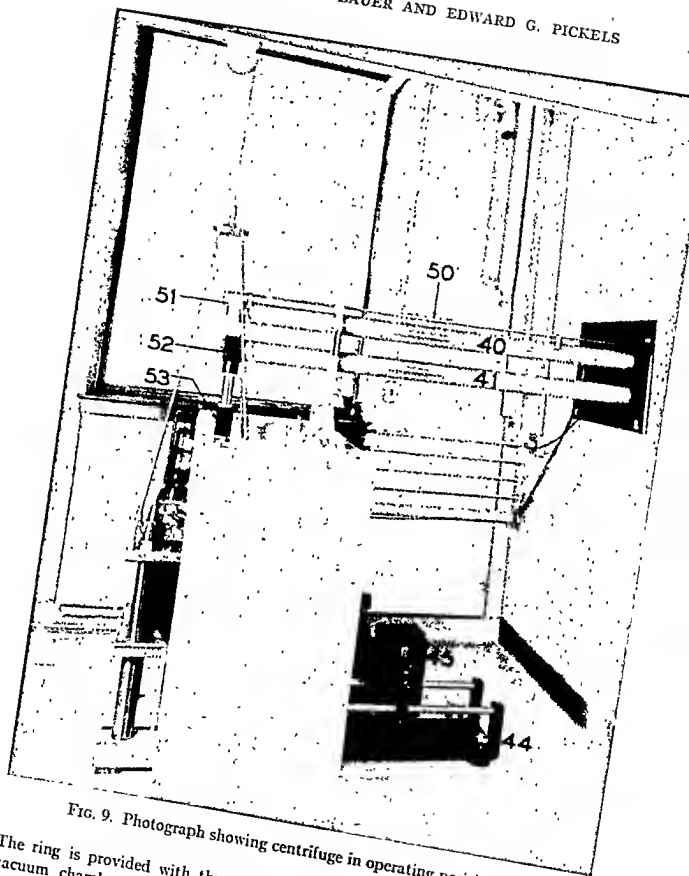


FIG. 9. Photograph showing centrifuge in operating position.

The ring is provided with three legs which rest on the elevated floor of the vacuum chamber. The ensemble is freely movable. When the top plate of the vacuum chamber is lowered into place for operation, the steel stem of

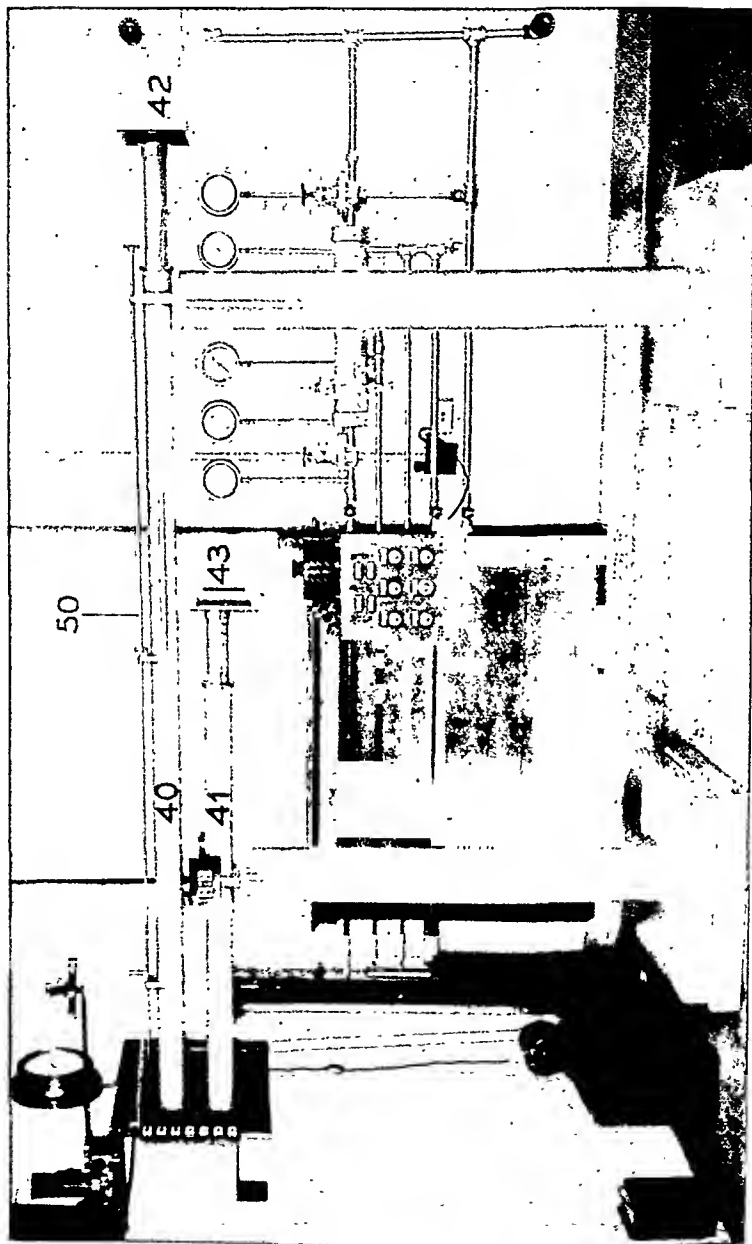


FIG. 10. Photograph showing camera ends and air control system.

the rotor (23, Figs. 3, 4) slips into the cup and projects into the mercury pool to a depth of about 3 mm. The cup and its support are shaken about by the stem of the rotor as the centrifuge is started, but as the speed increases and the motion becomes smooth and free from vibration, the ensemble is automatically shifted into a stationary position where the stem remains approximately centered in the cup and makes mechanical contact only with the mercury. Several other designs were tried and discarded because with them it was impossible to prevent the splashing out of mercury during operation. The successful application of this temperature-measuring device is made possible only because mercury fortunately possesses the three necessary properties, *viz.*, low viscosity, low vapor pressure, and good thermal conductivity. All temperature readings are taken with a galvanometer having a sensitivity of approximately $0.07^{\circ}\text{C. per mm. of scale deflection}$ when used with the thermocouples described.

Speed Measurements.—The speed of the rotor is measured with a stroboscope of a simple slotted disc type. The shaft of the stroboscope is connected with a revolution counter which is arranged to establish momentary electrical contact after every 250 revolutions of the disc. The electrical impulses can be used to activate an accurate timing clock or simply to illuminate a lamp which can be used as a signal when employing an ordinary stop watch. A synchronous, alternating current motor supplies a steady rotation to a pulley wheel by means of a rubber belt. This pulley wheel, fitted with a rubber rim, rotates against, and at right angles to, the stroboscope disc. By a simple screw arrangement, the wheel can be moved across the face of the disc to any desired setting, and consequently can be adjusted to give the disc a constant speed in any desired range.

Air Lines and Controls.—Successful operation of the centrifuge depends upon having approximately constant pressures in all the air lines supplying the various parts of the driving mechanism. The system of regulators and controls used is shown in Figs. 10 and 11. Air from the main line is first passed through a fine wire screen filter to remove any particulate matter which might interfere with the operation of the controls. The two reducing valves (56, 57, Fig. 11) generally known as Type 3Y^s have proved satisfactory. The first of these drops the head pressure (150 to 225 lb. per sq. in.) to about 100 lb. per sq. in. The second reduces the pressure to 60 lb. per sq. in., and holds it constant at that value as long as the setting of the control valve (58, Fig. 11) which serves the driving line is not varied over too wide a range. A steel chamber (59, Fig. 11), serving as an air reservoir, is inserted between the two reducing valves as an additional precaution against any slight fluctuations in the air flow to the centrifuge.

The purposes and characteristics of the four different lines leading to the centrifuge are as follows. The upmost line (60, Fig. 11) supplies air to the driving jets. An ordinary needle valve (58, Fig. 11) gives sufficiently sensitive adjustment of the driving pressure. The first gauge (61, Fig. 11) has a range of 0 to 60 lb. per sq. in., and is used only in connection with the main needle valve (58, Fig. 11).

^s Supplied by the Foster Engineering Company of Newark, N. J.

When driving pressures above 50 lb. per sq. in. are desired, as for instance, when a rapid acceleration of the centrifuge is needed, the lower valve (62, Fig. 11) is opened to admit the air from the main line and readings are taken from the coarser gauge (63, Fig. 11) which has a range of 0 to 300 lb. per sq. in., the finer gauge (61, Fig. 11) being first closed from the system with its stop cock (64, Fig. 11). Air for the supporting bearing is supplied through line 65, Fig. 11. It requires very critical adjustment and only a small flow of air; an especially sensitive valve (66, Fig. 11) is therefore used. Line 67, Fig. 11, furnishes pressure to a chamber from which lubricant is forced into the oil gland. This line requires the least critical control and therefore an ordinary small reducing valve (68, Fig. 11) serves very well. Gauges serving the air-bearing and oil lines (69, 70, Fig. 11) have a

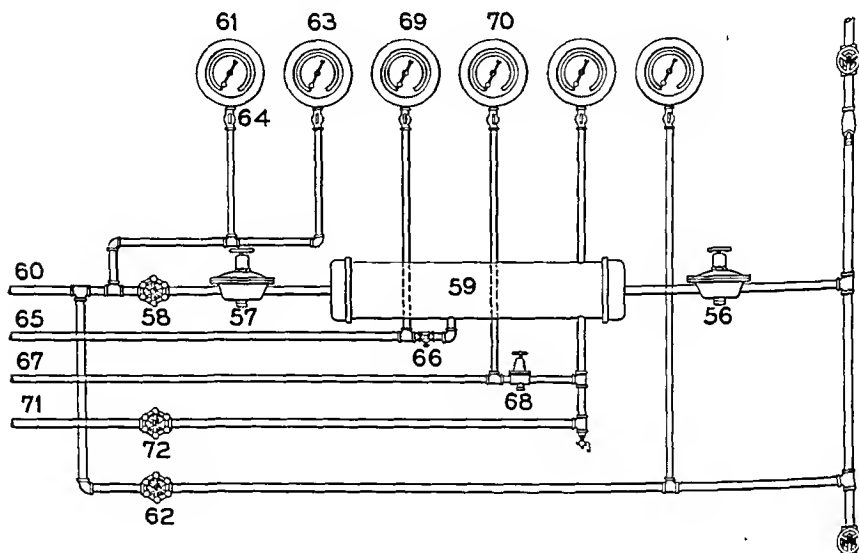


FIG. 11. Diagram of air controls.

range of 0 to 30 lb. per sq. in. Air line 71, Fig. 11, operates the reversing mechanism and is controlled by an ordinary needle valve (72, Fig. 11).

Operation Characteristics.—The operation characteristics of the air turbine drive as applied to a high speed vacuum centrifuge with a large and heavy circular rotor have already been fully described (20). These characteristics, in their essentials, have remained unchanged when the driving mechanism was adapted to the molecular ultracentrifuge with a lighter, oval-shaped rotor, driven at much higher speeds. The actual procedures involved in carrying out a centrifugation experiment are briefly as follows: The cell containing the material to be centrifuged is inserted into the rotor, and the edges of the sector in the cell are properly aligned with radii of the rotor. The rotor is then fastened to the drive shaft, the vacuum chamber is closed, the pressure in the lubricating system set at about 10 lb. per

sq. in., and the vacuum pump started. The vacuum pump is kept running throughout the experiment, and as soon as the pressure in the chamber reaches about 15 microns of mercury, the centrifuge itself is started. The supporting pressure is set at about $6\frac{1}{2}$ lb. per sq. in., which is just enough to lift the turbine and rotor so that they will turn freely. The driving pressure is then set at about 20 lb. per sq. in. and kept at that value for a few minutes until the rotor accelerates through a short period of precessional motions which occur at low speeds. As soon as the precessional motions cease, the driving pressure is usually raised to about 80 lb. per sq. in. and held there until the desired speed is reached. The pressure is then reduced to a value just sufficient to maintain this speed. To stop the centrifuge, the reversed air jets are put into operation and similar adjustments of the driving pressure are made as during the acceleration.

In addition to the precessional motions of the rotor at low speeds, there are slight vibrations, generated in the oil and air bearings, which are transmitted to all parts of the driving mechanism. In order to minimize these vibrations as the turbine accelerates, it is sometimes necessary to readjust the supporting pressure slightly. Characteristic movements of its gauge needle usually serve to indicate whether a raising or lowering of the pressure is needed. As the speed increases above several thousand revolutions per minute however, the motion becomes extremely smooth. As a matter of fact, ripples are not even visible on the surface of mercury contained in a dish placed on the plate supporting the oil gland.

With the driving pressure set at 80 lb. per sq. in., only about 12 minutes of acceleration have been necessary for the rotor to reach a speed of 60,000 R.P.M., and to maintain that speed a reduced driving pressure of about 18 lb. per sq. in. has been sufficient. No difficulty has been experienced in keeping the speed constant to within a fraction of 1 per cent. To bring the rotor to a full stop from 60,000 R.P.M. requires about 20 minutes.

DISCUSSION

Due to the fact that no special provision has been made for conducting away the small amount of heat generated by the frictional resistance of the residual air in the vacuum chamber, there is always a slow rise in the rotor temperature during a run, at least until a temperature equilibrium is established. With circular rotors such rises were previously reported by Biscoe, Pickels, and Wyckoff (18) as being negligible. These increases of temperature were found to be considerably more pronounced with the oval-shaped rotor operating at higher speeds in a vacuum of 10 to 15 microns of mercury usually obtained in the chamber with the Cenco megavac pump. Fortunately, these temperature rises have not been accompanied by temperature gradients sufficiently great to cause detectable convec-

tion currents within the fluids in the cell. This is demonstrated by the serial photographs in Fig. 12, recording the sedimentation of crystalline egg albumin in a 1 per cent solution centrifuged at a speed of 60,000 R.P.M., which corresponds to a centrifugal force of 260,000 times gravity. The intensity of a temperature gradient depends directly, of course, on the rate at which heat is being conducted through the rotor, and not necessarily on the rate at which the temperature of the rotor as a whole increases. As long as there are no serious convection currents to interfere with the sedimentation of

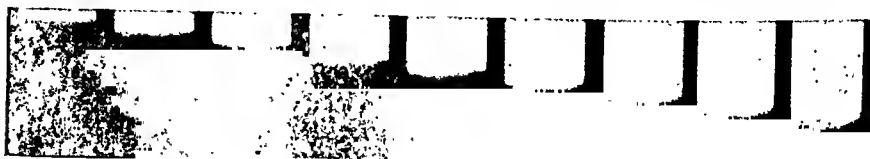


FIG. 12. Sedimentation of crystalline egg albumin in 1 per cent solution at a speed of 60,000 R.P.M.; photographs taken at 20 minute intervals.

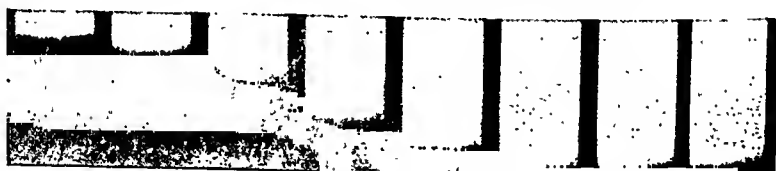


FIG. 13. Sedimentation boundaries in a mixture consisting of 0.3 per cent of normal horse serum globulin and 0.6 per cent of excelsin; centrifuged at a speed of 54,000 R.P.M.; photographs taken at 15 minute intervals.

molecules in the centrifugal field, and as long as the actual temperature of the rotor is known at all times during centrifugation, a rise of a few degrees in the rotor temperature is not a serious handicap. Recently the efficiency of the vacuum system has been improved by the addition of an oil diffusion pump, with which the pressure in the chamber is maintained at less than 1 micron of mercury. Preliminary experiments with the lower pressures have shown temperature rises of only 1 or 2°C. in the rotor during several hours' operation at high speed.

The work in developing the air-driven ultracentrifuge is greatly facilitated by the fact that many of the problems involved have

already been solved by Svedberg and his associates, and a number of the mechanical features which have been developed by them through years of painstaking research are readily adaptable to the new type of centrifuge described above.

SUMMARY

1. A description is given of the construction details and operation characteristics of an improved type of air-driven ultracentrifuge operating in vacuum and suitable for the determination of sedimentation constants of protein molecules.

2. The rotor of the centrifuge is made of a forged aluminum alloy; it is oval in shape, measures 185 mm. at its greatest diameter, and weighs 3,430 gm. It carries a transparent cell located at a distance of 65 mm. from the axis of rotation and designed to accommodate a fluid column 15 mm. high.

3. The rotor has been run repeatedly over long periods at a speed of 60,000 R.P.M., which corresponds to a centrifugal force of 260,000 times gravity in the center of the cell. At this speed no deformation of the rotor nor leakage of the cell has been observed.

4. The sharp definition of sedimentation photographs taken at high speed serves to indicate the absence of detectable vibrations in the centrifuge.

5. When a vacuum of less than 1 micron of mercury is maintained in the centrifuge chamber, the rise in the rotor temperature amounts to only 1 or 2°C. after several hours' run at high speed.

6. There has been no evidence of convection currents interfering with normal sedimentation of protein molecules in the centrifugal field.

7. A driving air pressure of about 18 pounds per square inch is sufficient to maintain the centrifuge at a steady speed of 60,000 R.P.M. With a driving pressure of 80 pounds per square inch, it can be accelerated to this speed in less than 20 minutes, and also brought to rest in about the same length of time by the application of the braking system.

8. The adaptation of Svedberg's optical systems to this centrifuge for photographically recording the movement of sedimentation boundaries is described.

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TISSUE CULTURE STUDIES ON BACTERIAL HYPERSENSITIVITY

IV. PROTECTIVE EFFECT OF IMMUNE PLASMA AGAINST THE DELETERIOUS INFLUENCE OF STREPTOCOCCAL EXTRACT ON HYPERSENSITIVE CELLS

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Crude hemolytic streptococcal extract in proper concentration exerts a comparatively greater toxic action on cells from guinea pigs infected with these microorganisms than on normal cells when studied by the tissue culture technique (1). That this action is relatively specific was established by comparing it with that of various other bacterial extracts and products; only those prepared from homologous strains or from strains in the same serological group C (2) exerted a specific inhibiting influence on the sensitive cells. It is not improbable that a guinea pig elaborates similar toxic substances in foci chronically infected with these microorganisms, yet in spite of persisting cellular sensitivity (1) during the chronic stage of the infection, the animals appear healthy, gain weight, and seem relatively unaffected by the large abscesses containing many living streptococci. This phenomenon might be explained by the presence in the blood of a factor capable of protecting the sensitive cells of infected animals from these hypothetical toxic substances; and if such a factor exists there, it might be subject to detection and analysis in tissue cultures.

The present communication reports experiments which test the validity of the foregoing hypothesis. Because agglutinins and precipitins had been detected throughout the course of this chronic streptococcal infection (1), the association of these antibodies with the hypothetical protective substance was also studied.

EXPERIMENTAL

Guinea pigs were infected with a group C (Lancefield) hemolytic streptococcus, strain K 104. The experimental methods, tissue culture media, type of observations, and quantitative formulae are similar to those detailed previously (1, 3). The plasmas from normal and infected animals are designated normal and immune, respectively. Comparative cytotoxic indices of bacterial extract for sensitive cells from infected animals when these cells were grown in normal plasma, with and without bacterial extract, and when grown in immune plasma, with and without bacterial extract, afford data for the analysis of the possible protective action of immune plasma. Thus in a typical experiment, 8 different conditions were imposed, and using 12 explants for each condition, a total of 96 was required. The rates of growth were determined in the following 8 experimental conditions.

1. Sensitive cells grown in normal plasma containing bacterial extract.¹ (Snb)²
2. Normal " " " " " " " " (Nnb)
3. Sensitive cells grown in normal plasma. (Sn)
4. Normal " " " " " " " " (Nn)
5. Sensitive cells grown in immune plasma containing bacterial extract. (Sib)
6. Normal " " " " " " " " (Nib)
7. Sensitive cells grown in immune plasma. (Si)
8. Normal " " " " " " " " (Ni)

The comparative cytotoxic index of bacterial extract for sensitive cells grown in normal media was determined by quantitative measurements of cellular migration in the first 4 experimental conditions, and is expressed by Formula 1 =

$$\frac{\frac{Snb}{Sn}}{\frac{Nnb}{Nn}}$$

The method for determining this index has been fully described (1, 3). Likewise, the comparative cytotoxic index of bacterial extract for sensitive cells grown in immune plasma was calculated by quantitative measurements of cellular migration in the 5th to 8th experimental conditions and is expressed in Formula

$$2 = \frac{\frac{Sib}{Si}}{\frac{Nib}{Ni}}.$$

¹ The final concentration of bacterial extract in the media was 1 to 6,000, a concentration which had only slight inhibitory effect on normal cells.

² The formulae express by means of letters the set up of each experimental condition. The capital letters "S" and "N" indicate sensitive and normal cells, respectively. The small letters "n" and "i" indicate normal and immune plasmas, respectively, while "b" indicates bacterial extract. For example: Snb shows that sensitive cells were grown in normal media containing bacterial extract, etc.

Obviously, if $\frac{\frac{Snb}{Sn}}{\frac{Nnb}{Nn}} = \frac{\frac{Sib}{Si}}{\frac{Nib}{Ni}}$ the value, or effect, of n (normal plasma) and

i (immune plasma) must be equivalent, because the other factors in the formulae are identical; hence there would have been no neutralization of the toxic effect of the bacterial extract b on the sensitive cells S . When, on the other hand, the value of Formula 2 is greater than that of 1, the factor responsible for this difference must be accounted for by the difference between the normal plasma n and the immune plasma i ; and the quantitative difference in the two comparative indices is an approximate expression of the relative protective action of the immune plasma i . The greater the value of Formula 2 over that of Formula 1, the proportionately greater is the neutralizing effect of the immune plasma. Microscopic appearances of the cells under the various experimental conditions also afford qualitative evidence of cellular injury, which can be correlated with the quantitative data.

RESULTS

In six experiments there was tested the capacity of immune serum to neutralize the toxic action of streptococcal extract on sensitive splenic cells, obtained from guinea pigs which had been infected for from 4 to 20 weeks. The degree of sensitivity of the cells grown in normal plasma as expressed by the comparative cytotoxic indices (Table I, column 8), is in inverse proportion to the index, which varied from 0.35 to 0.90.

When the same tissues, sensitive and normal, were tested with bacterial extract combined with immune plasma, the toxic action of the streptococcal extract was definitely less, as is indicated by the comparative cytotoxic indices which varied from 0.57 to 1.14 (Table I, column 9). Two of the indices (Experiments 213 and 223) were 1.12 and 1.14, respectively, which demonstrates complete neutralization of the specific toxic effect of the streptococcal extract by the two immune plasmas, respectively. In the other experiments a comparison of the two sets of indices indicates only partial neutralization by the immune plasmas. Qualitatively the microscopic appearances of the cells in the various flasks confirmed the quantitative data: the sensitive cells grown in a mixture of bacterial extract and immune plasma had a much healthier appearance than those grown with similar extracts and normal plasma. The plasmas in all experiments

except Nos. 213 and 253 were derived from infected and normal animals other than those from which the splenic explants were obtained.

Similar experiments were performed with plasmas from animals with acute infections of only 7 and 10 days' duration, a period before precipitins and increased agglutinin titers were demonstrable. These plasmas, however, were so intrinsically toxic that even without the addition of bacterial extract they greatly inhibited cellular activity of normal explants; hence it was impossible to test their capacity to neutralize the toxic effect of streptococcal extract on sensitive cells.

TABLE I

Column (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Experiment No.	Sensitive splenic explants (S) Duration of infection	Immune plasma (i) Duration of infection	Antibody titer of normal plasma (serum) (n)		Antibody titer of immune plasma (serum) (i)		Comparative cytotoxic index of bacterial extract in normal plasma $\frac{Snb}{Sn}$ $\frac{Nnb}{Nn}$	Comparative cytotoxic index of bacterial extract in immune plasma $\frac{Sib}{Si}$ $\frac{Nib}{Ni}$	Degree of neutralization by immune plasma of specific cyto- toxicity of streptococcal extract
			Agglu- tinin	Precip- itin	Agglu- tinin	Precip- itin			
	<i>wks.</i>	<i>wks.</i>							
213	10	10	40	—	320	+	0.90	1.12	Complete
219	7	9	Not done	—	Not done	+	0.48	0.57	Partial
223	4	4	40	—	640	+	0.69	1.14	Complete
235	6	6	160	—	320	+	0.55	0.73	Partial
253	21	20	20	—	1,280	+	0.35	0.68	"
254	14	14	10	—	640	+	0.42	0.67	"

Antibody Content of Normal and Immune Sera.—Agglutinin titers and precipitin reactions, determined as previously described (1), are shown in Table I. None of the normal sera and all of the immune sera contained precipitins. The agglutinin titers of the normal sera varied from 10 to 160 and of the immune sera from 320 to 1280. The normal serum with an agglutinin titer of 160 is abnormally high since the average titer for 21 normals (1) was found to be 43. Possibly this one high titer was due to a previous spontaneous infection with hemolytic streptococci, a type of infection very common among ordinary stocks of these animals.

In Table III these comparative values are recorded for each of the six experiments. The close correspondence of the respective values in the two columns of indices shows that the plasmas themselves did not exert either a selective toxic or stimulating effect on either the normal or sensitive tissues. These data strengthen the conception that some factor in the immune plasma neutralizes a component of the bacterial extract that is toxic for sensitive cells.

Failure to Neutralize with Immune Serum the Skin Reacting Substances in Bacterial Extract.—Various combinations of bacterial extract and immune serums containing both precipitins and agglutinins were tested on streptococcal infected animals showing cutaneous hyper-reactivity to bacterial extract. Some of the combinations of bacterial

TABLE III
Comparative Initial Growth Indices in Normal and Immune Plasmas

Experiment No.	Comparative initial growth index in normal plasma $\frac{S_n}{N_n}$	Comparative initial growth index in immune plasma $\frac{S_i}{N_i}$
213	0.91	0.96
219	1.65	1.62
223	1.31	1.44
235	0.68	0.67
253	0.96	0.91
254	1.13	1.12

extract and immune serum were injected soon after mixing, others after incubating for 2 hours, still others after incubating for 2 hours, storing overnight in the ice box, and removing the precipitate. In no test was there evidence that the substance inducing skin reactions was neutralized by the immune sera. This suggests either that different substances in the extracts are responsible for eliciting cutaneous reactions and for injuring cells in tissue cultures, or that the mechanism of responses are different. It is even possible that a mixture may have to be quite exactly balanced for neutralization to be demonstrable *in vivo*.

DISCUSSION

A guinea pig inoculated with some strains of group C hemolytic streptococci passes through several stages that have certain analogies

to those seen in some human infections. For a period of about 2 weeks after inoculation the animal appears acutely ill, has fever, and loses weight, and during the first part of the infection there are marked local signs of inflammation at the site of inoculation. Then the local lesion breaks down, discharges its contents, and heals; but the satellite lymph nodes and those more distant become the sites of chronic lesions in which large numbers of streptococci are harbored. The animal develops hypersensitivity to products of these streptococci, hypersensitivity that can be demonstrated in the skin, and at the same time *in vitro*, since sensitive mesenchymal cells are specifically injured in tissue cultures containing extracts of the streptococci. After the 2nd week, however, even though the animal's tissues are still sensitive to streptococci which are growing in large numbers in its body, the guinea pig thrives, is fever-free, and shows no obvious general toxic manifestations. Whatever may be the mechanism that protects the animal, this mechanism obviously neither eliminates the irritating streptococci nor renders the cells less susceptible to the streptococcal toxic products. It seemed of more than passing interest that coincidentally with the appearance of circulating antibodies in the blood stream the animal's general condition improves; and this suggested that some humoral substance might protect the sensitive cells from toxic factors elaborated in the areas of chronic focal infection.

As a matter of fact, the present study shows that plasmas from animals infected with group C streptococci do neutralize the factor in streptococcal extracts which is responsible for the toxic action on sensitive cells in tissue cultures; moreover, this neutralizing effect is roughly quantitatively parallel with the concentration of agglutinins in the respective sera. It is as yet impossible to tell the nature of this neutralizing substance, for the method of demonstrating it is different from those usually employed for detecting antibodies. As these susceptibilities, toxic components, and neutralizing factors are all present in the animal body, it would appear that we have a mechanism whereby the animal's tissues are protected even in the presence of a toxic agent, and hence the chronic infection is well tolerated. If, on the other hand, the neutralizing factor is insufficient in amount to render the toxic substances entirely harmless, this condition of affairs may account for a continuing effect of the focal infection on distantly

situated sensitive cells. Since our experimental animals have been usually efficient in producing this neutralizing factor, few opportunities for studying this phase of infection have presented themselves. Experiments are in progress on inhibition of antibody formation which may throw further light on this problem.

SUMMARY

1. Plasmas from guinea pigs, chronically infected with group C hemolytic streptococci, neutralize the components of bacterial extract which exert a marked toxic action on hypersensitive cells *in vitro*.

2. The neutralizing capacity of these immune plasmas is relatively specific for the bacterial extract, and is not due to a variable non-specific effect on normal or hypersensitive tissue cells.

3. A rough correlation between the agglutinin titer and the relative neutralizing capacity of immune plasma suggests that the latter may be a manifestation of antibody action.

4. The tolerance by guinea pigs of chronic hemolytic streptococcal lymphadenitis is explainable, at least in part, by the neutralizing capacity of their plasmas, since such soluble bacterial products as may be absorbed from infectious foci would probably be neutralized before they could exert a deleterious influence on the hypersensitive cells of the animals.

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STUDIES ON THE SEROLOGICAL TYPING OF STREPTOCOCCUS HEMOLYTICUS*

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Two factors involved in the development of rheumatic activity have seemed especially significant. Recent emphasis has been placed on the character of the immune response of the rheumatic subject to respiratory infection with hemolytic streptococcus. Swift and Hodge (1) have found a delay in the appearance of anti-M precipitins in rheumatic patients as compared with non-rheumatic subjects. Our findings (2) have indicated that initiation of the rheumatic attack is associated with an immune response which is atypical in several respects.

The other factor in which we have been particularly interested is the nature of the infectious agent. The biological character of the infecting strain has seemed to determine in part whether or not the quiescent rheumatic subject develops a recrudescence following pharyngitis with hemolytic streptococcus (3). Preliminary observations have suggested that serological classification of these organisms may make it possible to identify the strains which are effective in initiating rheumatic activity from year to year in different sections of the world. The present studies deal first with the methods devised for simplifying the serological classification of hemolytic streptococcus and second with the results of typing of these organisms associated with pharyngitis in rheumatic subjects.

A systematic classification of hemolytic streptococcus of human origin was begun by Dochez, Avery and Lancefield (4). These authors

* The work reported in this communication was carried out under The W. K. Kellogg Foundation Fund.

found that 125 strains associated with respiratory infections could be subdivided by means of the agglutination reaction into at least four distinct biological types. Lancefield (5) demonstrated the presence of a type-specific substance of protein character in hemolytic streptococcus and developed a method for classification based on the precipitin test. Coburn and Pauli (6), applying the precipitin method of Lancefield to strains of hemolytic streptococcus recovered from the throats of rheumatic subjects during pharyngitis, found a number of different serological classes of hemolytic streptococcus. Complete serological classification of these organisms was impracticable at that time because of the cross-reactions due to the high concentration of carbohydrate antibody (anti-C) in some of the rabbit antisera (6). The removal of anti-C by absorption was attempted, but had to be abandoned as repeated absorptions with a crude fraction of streptococcus carbohydrate diluted the content of precipitin to such a degree that the sera became useless.

A method recently developed by Griffith (7) in which slide agglutinations are performed with absorbed antisera has made it possible to distinguish more than twenty serological types of hemolytic streptococcus. This method permits type identification in a large majority of organisms and should be invaluable in epidemiological studies were it not handicapped by the technical difficulties in the absorption procedure. This step in Griffith's technique consists in absorbing each antiserum with a suspension composed of organisms from all of the heterologous types. The possibility of under- or over-absorption presents a constant danger to most workers. The present authors have therefore attempted to eliminate this difficulty in order that Griffith's method may have the widespread application which it deserves.

Comparative tests of Griffith's slide agglutination and Lancefield's precipitin methods have shown general agreement (8). In Lancefield's opinion both of these phenomena represent reactions between anti-M and the type-specific M substance. Since the presence of anti-C in rabbit sera is known to interfere (6) with satisfactory typing by the precipitin method, it seemed likely that it might also be responsible for cross-reactions in Griffith's method. The present paper deals with observations on typing with rabbit antisera from which anti-C had been removed.

Technique

1. *Preparation of Broth Used in Making Antigens for Immunization.*—2 pounds of chopped fresh beef hearts are placed in 2 liters of distilled water, extracted for 1 hour and refrigerated overnight. The mixture is hoiled for 5 minutes, filtered through cheese cloth and the broth heated again to boiling. The pH is adjusted to 8.0. After bringing to hoil, the volume of 2 liters is restored with hoiling water. The pH is again checked. After 2 minutes of hoiling, 20 gm. of neopeptone and 8 gm. Na_2HPO_4 are added. The broth is autoclaved for 25 minutes at 15 pounds pressure.

2. *Preparation of Broth Used in Making Bacterial Suspensions.*—400 gm. of fresh heef heart are minced. To this are added 1 liter of tap water, 0.5 gm. Na_2HPO_4 . This is made alkaline to litmus with $\frac{1}{2}$ normal sodium hydroxide. The mixture is heated to 70–80°C. for 5 minutes. After cooling to 42°C., bacto-trypsin (Difco) is added, 10 cc. per liter. This is incubated at 45°C. from 1½ to 2 hours depending on the activity of the trypsin. Digestion is stopped when the hiuret test becomes positive. The medium is acidified with concentrated HCl until it is distinctly acid to litmus. The medium is then hoiled for a total of 20 minutes. At the end of 10 minutes of hoiling, the acidity to litmus is tested and more HCl added if necessary. The medium is then filtered through muslin and the pH of the broth adjusted to 7.8. Sodium chloride is added to a concentration of 0.25 per cent and then calcium chloride to 0.13 per cent. The broth is then hoiled for 5 minutes and filtered while hot through a fine filter paper (Eimer and Amend No. 23154). The filtrate is steamed in the Arnold sterilizer for 1 hour and for ½ hour on 2 successive days.

3. *Preparation of Antigen for Immunization.*—1½ liters of neopeptone huffered broth (described under 1 above) are inoculated with 5 cc. of a 5 hour seed culture (purity of culture being checked by Gram stain and plating). After 15 hours incubation at 37.5°C., the culture is cooled under running tap water and immediately centrifuged. The broth is decanted and the organisms are washed three times in 250 cc. normal saline. After the third washing, the bacteria are suspended in 250 cc. normal saline and immediately heated for 1 hour at 56–58°C. After centrifuging again, the killed organisms are suspended in 100 cc. of saline containing merthiolate (1 in 10,000). The vaccine is then standardized in concentration of 1, 2 and 5 billion organisms per cc.

4. *Immunization Procedure.*—Young male brown rabbits weighing 4 to 6 pounds are selected for immunization. A preliminary bleeding is made for control purposes. Vaccine is administered according to the following dosage.

1st week

1st day subcutaneously	0.5 cc. of 5 billion organisms per cc.
2nd " "	0.5 " " 5 " " " "
3rd " "	0.5 " " 5 " " " "
4th " intraperitoneally	1.0 " " 1 " " " "
5th " "	1.0 " " 1 " " " "
6th " "	1.0 " " 1 " " " "

2nd week

1st day intravenously	0.5	"	"	1	"	"	"	"	"
2nd " "	0.5	"	"	1	"	"	"	"	"
3rd " "	1.0	"	"	1	"	"	"	"	"
4th " "	1.0	"	"	1	"	"	"	"	"

3rd week

1st day intravenously	2.0	"	"	1	"	"	"	"	"
2nd " "	2.0	"	"	2	"	"	"	"	"
3rd " "	1.0	"	"	1	"	"	+	0.05 cc. of living culture*	
4th " "	1.0	"	"	1	"	"	+	0.05 " " " "	"

4th week

1st " "	1.0	"	"	1	"	"	+	0.1	" " " "
2nd " "	1.0	"	"	1	"	"	+	0.1	" " " "
3rd " "	1.0	"	"	1	"	"	+	0.1	" " " "
4th " "	1.0	"	"	1	"	"	+	0.1	" " " "

5th week

1st day—trial bleeding and testing.

2nd day—bleeding if titer is satisfactory. After large amounts of blood are taken, a 5 per cent glucose infusion is given and rabbits are allowed to rest for 4 or 5 weeks, after which they are given one series intravenously of vaccine (1 or 2 billion organisms per cc.). Upon testing, it will be found that in most instances the titer has risen, and the rabbits are then suitable for further bleeding.

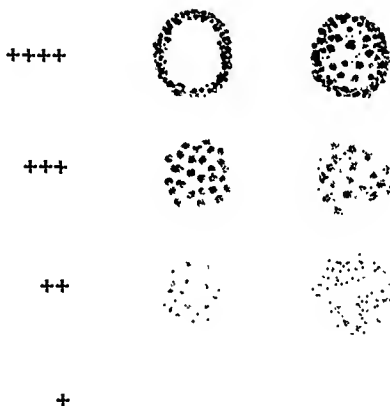
If the titer at first testing is found too low, further immunization is done for 2 weeks. Should the rabbits not respond, they are discarded and new rabbits immunized.

* All living cultures are washed three times and made up to original volume with normal saline.

5. *Technique of Slide Agglutination.*—The technique for testing the antisera and for identifying the type of unknown organisms is the same. We have followed Griffith's method in detail. 10 cc. of a 15 hour trypsin broth (described above under 2) culture are thoroughly mixed in a 10 cc. pipette. The culture is then centrifuged and most of the broth decanted. The organisms are suspended evenly in the remaining broth (approximately 0.1 to 0.2 cc.) by repeated aspiration into a capillary pipette. It is important that the suspension be homogeneous. A small drop of this bacterial suspension is placed on a slide with the capillary pipette and a tiny loopful (nichrome wire gauge 34, diameter of loop 0.5 mm.) of rabbit antiserum in the appropriate dilution is mixed with the suspension on the slide. The slide is rotated and readings are made immediately with a hand lens (magnification 6×). The results are read as follows:

- ++++ drop containing large clumps which tend to settle around the periphery of drop, making a heavy outline.
- +++ similar to the above except that the clumps are not so heavy.
- ++ many fine clumps of agglutinated organisms.
- + few small clumps of agglutinated organisms scattered throughout the suspension.
- 0 homogeneous suspension—no clumps.

Examples of each are illustrated in Fig. 1.



Negative

FIG. 1

TABLE 1
Slide Agglutination Reactions between Crude Rabbit Antisera and Suspen-

Rabbit antisera (dilution 1:10)		Suspensions of hemolytic streptococcus-														
Types	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1	++++															
2		++++														
3			++++													
4				++++								++				
5	++				++							+++				
6						++++					+					
7			±				++++									
8			++					++++								
9			+						++++							
10			±							++++						
11											++++					
12			++									++++				
13			++									++++	++++			
14			++										++++	++++		
15														++++	++	
16																
17																
18			+													
19			++	+									+++			
20																
21																
22			++										+++			
23			++										++++			
24			++													
25																
26													++			
27			+													
28																

The blank spaces in the tables indicate negative results.

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The above technique is essentially that used by Griffith. However, two special precautions have been introduced in order to minimize cross-reactions without resorting to absorption. First, the cultures used for immunization were washed in saline at least three times to free them from broth and to remove any carbohydrate which might adhere to the surface of the bacterial cells. Second, the rabbits were bled as early as possible in the course of immunization (4th week) in order to obtain sera with minimal anti-C titers. The agglutination reactions between the 28 crude antisera and Griffith's 28 type strains are presented in Table I.

There are three points of interest in Table I. First, five of the antisera were type-specific: that is, they agglutinated only the suspension of homologous organisms (*e.g.* types 1, 2, 7, 11 and 21).

TABLE II
Relation between Anti-C Content and Intensity of Cross-Reactions in Rabbit Antisera

Unabsorbed rabbit antisera	No. of cross-reactions with cell suspensions of heterologous types	Precipitin reactions with streptococcus carbohydrate dilution 1:10,000	
		2 hrs.	24 hrs. (centrifuged)
Type 1	0	0	(0)
Type 2	0	0	(0)
Type 15	Four (++)	+	(++±)
Type 23	Four (++++)	+++	(++++)

Second, a number of antisera showed cross-reactions of varying degree with suspensions of heterologous types; slight (type 3), moderate (type 22), marked (type 13). Finally, four of the bacterial suspensions (types 3, 12, 23, 24) were agglutinated by a number of antisera.

The Relation of Anti-C to Cross-Reactions

In order to test the possibility that cross-reactions might be due to the presence of anti-C in the sera, the concentration of this antibody was determined in antisera of the various types. The test was performed by adding 0.2 cc. of crude antiserum to 0.2 cc. of purified carbohydrate¹ diluted 1:10,000. Four observations of precipitin

¹ The streptococcus carbohydrate fraction used throughout this study was kindly given to us by Dr. Michael Heidelberger and Dr. Forrest E. Kendall. The method of preparation is to be reported by them.

TABLE III
The Effect of Repeated Absorptions with Carbohydrate on the Specificity of Slide Agglutination

	Type No.	Precipitin reaction with carbohydrate			Slide agglutination reactions with suspension of types			Reactions of unabsorbed controls diluted with saline
		3	12	24	3	12	24	
Reactions of unabsorbed undiluted sera	3 12 24	0 ++ +	± ++ ++	±± ++ ++	++ ++ ++	++ ++ ++	++ ++ ++	Same as original sera
Reactions after first absorption of crude sera	3 12 24	0 0 0	0 ± ±	± ++ ++	++ ++ ++	++ ++ ++	++ + ++	
Reactions after second absorption	3 12 24	0 0 0	0 0 0	0 ± +	++ ++ ++	++ ++ ++	0 + ++	
Reactions after third absorption	3 12 24	0 0 0	0 0 0	0 0 ±	++ + ++	± ++ ++	± + ++	Sera showed slight diminution in strength of heterologous reactions
Reactions after fourth absorption	3 12	0 0	0 0	0 0	++ +	0 ++	± +	
	24	0	0	±	0	++	++	

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formation were generally made: immediately, after 2 hours in 37° water bath, after refrigeration overnight and after centrifuging. A sample protocol is presented in Table II.

As illustrated in Table II, type-specific antisera were free of anti-C and the sera which gave cross-reaction contained anti-C. This was observed throughout the 28 types. In general there was a close correspondence between the concentration of anti-C and the intensity of cross-reactions.

The sera containing anti-C were absorbed with carbohydrate. The number of absorptions necessary was found to vary directly with the concentration of anti-C. However, repeated absorptions caused no apparent diminution in the strength of the type-specific agglutination reaction. This is shown in Table III. From this it is seen that repeated absorptions progressively diminished the cross-reactions without at all weakening the homologous reactions.

The next step consisted in a simplification of the absorption technique. It was found that by diluting the serum 1:5 and absorbing

TABLE IV
The Elimination of Cross-Reactions

Treatment of type 23 serum	Reactions with carbohydrate				Reaction with suspension types			
	+ + + + + ± (++++)				3	12	23	24
Unabsorbed, 1:10	+	+	+	+	+	++	++++	++++
Diluted 1:5 and absorbed with equal volume of carbohydrate 1:5,000. Final dilution 1:10	0	0	0	(0)	0	0	++++	0

with an equal volume of carbohydrate 1:5,000 a single absorption was usually sufficient to eliminate cross-reactions. This is illustrated by Table IV.

It is to be observed from Tables III and IV that the absorption of anti-C was paralleled by the disappearance of cross-reactions, irrespective of the number of absorptions and the dilutions employed.

Removal of anti-C in no case weakened the type-specific reaction. The following procedure has been adopted as standard in this laboratory. Antisera which contain no anti-C and give no cross-reactions are simply diluted 1:10 without absorption. The antisera which contain anti-C and give cross-reactions from + to +++ are diluted 1:5 and then absorbed with an equal volume of carbohydrate, 1:5,000. After the precipitate is removed, the supernatant is a type-specific serum and has a final dilution of 1:10. The antisera which contain a great deal of anti-C and give strong cross-reactions are diluted 1:2.5 and absorbed with an equal volume of carbohydrate 1:5,000. The supernatant is then reabsorbed with an equal volume of carbohydrate 1:10,000. This doubly absorbed serum is either type-specific or may give insignificant cross-reactions which can be removed if desired by further absorption. The results of the slide agglutination tests with the antisera treated in this way are presented in Table V. The final dilution of all sera is 1:10.

Table V shows that after the sera had been absorbed with carbohydrate, only four cross-reactions persisted. Two of these were readily removed with one more absorption. However, it was found that type 17 serum freed of all anti-C gave a cross-reaction with type 23 organisms, and that type 23 serum freed of all anti-C gave a cross-reaction with type 17 organisms. No other sera gave cross-reactions after anti-C had been completely removed. It appeared from this slide agglutination that these two types were antigenically indistinguishable. Two further attempts to differentiate these sera were made: First, each serum was absorbed with the homologous and the heterologous organisms and then tested for agglutinins to types 17 and 23. Again the two sera were indistinguishable. Finally sera of the two types were tested for precipitins to type-specific M substance (5). No differences could be detected between types 17 and 23.

With the procedure as outlined it is possible to obtain strong agglutinating antisera in 1 to 10 dilution that are, for practical purposes, type-specific. Such sera may be pooled (type 1 to 6, 7 to 12, etc.). With this preliminary step the organism is found to be one of five groups. Type identification may then be made quickly and simply.

V

pneumococcus—Griffith's 28 type strains

[illegible]

TABLE VI
Slide Agglutination Reaction of Bacterial Suspensions Types 12 and 24

Type of antiserum.....	3	4	5	8	9	12	13	15	19	20	22	23	24	25	26	27	28	All others
Type 12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Organisms grown in standard way	++	+++	0	0	+++	++++	++++	++	+++	0	+++	++++	0	0	++	0	0	0
Type 12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Organisms grown in presence of anti-C	0	0	0	0	0	++++	0	0	0	0	0	0	0	0	0	0	0	0
sheep serum	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Type 24	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Organisms grown in standard way	++	++	+++	++	++	++	+++	+++	++	++	++	+++	+++	++	++	++	++	++
Type 24	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Organisms grown in presence of anti-C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
sheep serum	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

As was seen in Table I, some of the bacterial suspensions showed a tendency to be agglutinated by many antisera of heterologous types. This was most marked in the case of type 12 and 24 organisms. One possible explanation for this phenomenon is that these bacterial cells are rich in carbohydrate. With a view to inhibiting carbohydrate formation these organisms were grown in broth containing 10 per cent sheep antistreptococcus serum. This sheep serum had a high titer of antibody to the carbohydrate; that is, it gave a ++++ reaction uncentrifuged. Daily transfers of minimal inoculations of an 18 hour culture were made for 2 weeks. At the end of that time the suspensions of organisms showed a tendency to become granular but were nevertheless readily typed. The resulting improvement in specificity of one of these types is shown in Table VI.

When grown in the usual manner these organisms consistently reacted with the sera of the heterologous types containing anti-C. However, the bacterial cells prepared by prolonged culture in anti-C sheep serum broth reacted only with the homologous rabbit antiserum. The authors are now attempting to obtain antisera which give only type-specific reactions by immunizing with organisms in which the formation of carbohydrate has been repressed.

Results of Typing 1935-1936 Cultures

147 strains of hemolytic streptococcus were obtained from throat infections between September, 1935, and August, 1936. Of these 107 were identified and the types were distributed as follows:

Type.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14
No. of cultures.....	3	2	9	26	4	5	4	0	2	0	0	0	17	0
Type.....	15	16	17*	18	19	20	21	22	23	24	25	26	27	28
No. of cultures.....	0	6	4	4	1	0	2	15	0	0	1	0	1	1

* These four suspensions gave strong agglutination reactions with type 17 antiserum, weak reactions with Griffith's provisional type 23 antiserum and are classified as type 17.

Most of these strains were sent to Dr. F. F. Griffith for independent classification. Using his own suspensions and antisera, he checked every typing but one.

40 strains could not be typed by the standard procedure, for two reasons: (a) Certain strains were too granular for the slide agglutination technique. This may have been due to some unexplained characteristic of particular batches of trypsin broth, or to some characteristic of the organism. A tendency to be granular was especially noticeable in organisms which formed extremely matt colonies. The difficulty could be overcome in some instances by rapid passage through trypsin broth (every 3 hours), followed by plating on blood agar. A smooth colony was then selected for replanting in broth. Dr. Griffith succeeded in identifying twelve of these refractory strains. (b) Four other strains gave excellent suspensions but were not agglutinated by any of the available antisera. It is possible that these organisms either lack specific M substance, or belong to types as yet unidentified.

The Relation of the Types to Rheumatic Recrudescences

The above strains were obtained from throat infections in three groups of patients, all resident in or near New York City. One group of ten had scarlet fever; the predominant organism was type 3. Another group consisted of nurses with tonsillitis; these yielded a variety of types. The largest group consisted of rheumatic subjects from whom three predominant types were recovered: 13, 4 and 22.

It is our impression that the effects of strains 22 and 13 on rheumatic subjects were clinically different. Type 22 seemed relatively ineffective in initiating rheumatic activity. In contrast, most of the recrudescences following pharyngitis with type 13 were especially severe (2). It will be of interest to learn whether these differences between types are constant from year to year and whether new types will appear in predominance. Similar studies in other environments should furnish valuable information.

DISCUSSION

Lancefield's (9) serological classification of the groups of hemolytic streptococcus makes it possible to identify strains pathogenic to man (group A). Further subdivision in this group A can be accomplished with Griffith's technique. It seems to us that some of the types originally defined by Griffith must be eliminated. Four of these are 7, 16, 20 and 21 which do not belong to group A and should be

classified in their appropriate groups. Type 23 in our experiments is indistinguishable from Type 17 and we believe that both have the same type-specific antigen. As new types appear in human infections their group should be determined and those which belong to group A should be given type numbers in Griffith's series, beginning with those numbers which have become available by the above or any future elimination of provisional types.

The slide agglutination reaction, like the precipitin reaction, depends on the presence of either or both of two substances. Group-specific reactions occur in the presence of C substance and its antibody and type-specific reactions occur in the presence of M substance and its antibody. These two antigens must, therefore, be present on the surface of the bacterial cells. There is another antibody which we know is present in high titer in our rabbit antisera but which does not take part in the slide agglutination reaction. This is anti-P. The simplest explanation for the absence of reaction between bacterial suspensions and anti-P is that P is not present on the surface of the bacterial cell.

SUMMARY

The cross-reactions which interfere with satisfactory serological identification of hemolytic streptococcus are due to anticarbohydrate in the sera used for typing.

This antibody can be removed easily by absorption with purified streptococcus carbohydrate, and type identification is then readily established.

The serological classification of hemolytic streptococcus from throat infections contracted in New York during 1935 and 1936 showed the predominance of types 4, 13 and 22. Type 13 appeared to be the most serious in initiating rheumatic activity during this period of observation.

The authors are deeply indebted to Dr. F. F. Griffith of the British Ministry of Health for invaluable assistance and to Dr. Forrest E. Kendall for advice throughout this study.

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THE COAGULATION OF BLOOD BY SNAKE VENOMS AND ITS PHYSIOLOGIC SIGNIFICANCE

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It has recently been demonstrated (5a) that certain proteolytic enzymes can cause the coagulation of blood or plasma by either of two mechanisms. Trypsin acts on prothrombin to form thrombin, and is thus the counterpart of the physiologic calcium-platelet system. Papain, however, acts directly on fibrinogen to form a fibrillar gel resembling fibrin, and is thus the counterpart of thrombin. In view of these findings, it was suggested that a calcium-platelet mixture (or a calcium-tissue extract mixture) contains a proteolytic enzyme which, like trypsin, reacts with prothrombin to form thrombin. It was further suggested that thrombin itself is a proteolytic enzyme analogous to papain which hydrolyzes fibrinogen to form an insoluble split product, fibrin. The kinetics of thrombin formation and of the resultant coagulation were found to be in accord with the thesis that physiological coagulation involves these two consecutive enzyme reactions (3, 4). Moreover, an analogy for this hypothesis was seen in the activation of chymotrypsinogen by trypsin to form a new enzyme, chymotrypsin (11).

Snake venoms are known to affect the phenomenon of blood coagulation profoundly. Some inhibit the clotting process when added to blood *in vivo* or *in vitro*, a few have no significant effect, and a large number have a marked coagulative action on whole blood, plasma, or fibrinogen. Most of those who have concerned themselves with the mechanism of this coagulation (1, 6, 9, 10, 13, 14, 21) have found the venoms to act directly on fibrinogen. Only in an early paper by Mellanby (15) does one find the suggestion that some other process may be involved. On adding the venom of either *Echis carinata* (Indian

viper) or *Notechis scutatus* (Australian tiger snake) to a solution containing prothrombin and fibrinogen, Mellanby noted that the coagulating activity of the mixture steadily increased, even after coagulation had occurred. He concluded that the venom constituted a kinase which functioned like a platelet suspension; and since platelets are effective only in the presence of calcium, he was constrained to assume that the prothrombin-fibrinogen mixture contained calcium as part of a non-ionizing complex with the protein, and that this calcium acted in conjunction with the venom kinase to convert prothrombin to thrombin. Later workers have been almost unanimous in considering these coagulant venoms to act directly on fibrinogen.

In view of the coagulative action of trypsin and papain already cited, and since most snake venoms are known to contain proteolytic enzymes (6, 14), it becomes of obvious interest and significance to ascertain whether the coagulative action of snake venoms depends on their enzyme content, and whether these venoms are of two types: one type which, like trypsin and like the calcium-platelet system, acts on prothrombin to form thrombin; and one which, like papain and thrombin, acts directly on fibrinogen to form fibrin. The experiments here reported indicate that such is, indeed, the case.

*Methods and Materials*¹

Fibrinogen.—Citratd plasma was centrifuged at high speed and passed through a Berkefeld filter in order to remove platelets. The filtrate was precipitated with 1.5 volumes of saturated NaCl at room temperature. The precipitate was collected by centrifugation, the fluid discarded, and the glutinous protein precipitate allowed to express free fluid for half an hour at room temperature. It was then redissolved in a minimum volume of water, and reprecipitated by the addition of saturated NaCl (1.5 times the volume of water used for resolution). A trace of citrate was added to the water in order to prevent coagulation. Three such precipitations usually sufficed to yield a fibrinogen which did not coagulate on the addition of calcium and lung extract, but was promptly coagulated by thrombin. The final solution was rendered isotonic with blood by appropriate dilution before use.

¹ The venoms used in these experiments were obtained through the courtesy of Dr. John Reichel and Dr. Thomas S. Githens of the Mulford Biological Laboratories, Glenolden, Pennsylvania. The fresh citrated horse plasma used in these experiments was furnished by the same laboratories.

Crude Prothrombin.—This was prepared by precipitation with acetic acid as described by Mellanby (16). One volume of plasma was diluted with 15 volumes of cold water and precipitated by the addition of approximately $\frac{1}{10}$ the original plasma volume N/1 acetic acid. The precipitate was collected by centrifugation, and contained fibrinogen, prothrombin, euglobulin, and some substance with a platelet-like action. It was then redissolved in 0.85 per cent NaCl and freed of fibrinogen by heating at 56°C. for 3 minutes (3). Because the resultant solution contained either traces of platelet derivative or a cephalin-like plasma factor, it was activated to thrombin by calcium alone.

Pure Prothrombin.—In order to prepare a "pure" prothrombin solution, which would not be activated to thrombin by either calcium alone or tissue extracts alone, plasma was passed through a Berkefeld filter prior to its dilution and precipitation with acetic acid, and the resultant precipitate was then extracted with $\text{Ca}(\text{HCO}_3)_2$ by the Mellanby (16) technic. The final solution, rendered isotonic by the addition of $\frac{1}{10}$ volume of 17 per cent sodium chloride, was a highly active prothrombin preparation which contained no fibrinogen, and which rapidly evolved large amounts of thrombin on the addition of $\frac{1}{10}$ volume of 1 per cent CaCl_2 and cephalin (or lung extract). The addition of calcium alone had either no demonstrable effect, or caused a very slow elaboration of minute quantities of thrombin, less than 2 per cent of the amount elaborated from the same prothrombin in the presence of an adequate amount of cephalin or tissue derivative.

Lung Extract.—Fresh rabbit lung tissue was washed as free as possible of blood and expressed in a large volume of 0.85 per cent NaCl. The minced tissue remained reactive for months if it was kept frozen in a sealed container over solid CO_2 .

Quantitative Evaluation of Thrombin Activity.—This was accomplished by graphic interpolation on a curve correlating thrombin concentration and coagulation time (3).

Determination of Proteolytic Activity.—The proteolytic activity of the several venoms was determined with gelatin, using a modification of the Gates (7) technic. In this simple and accurate method a piece of exposed and fully developed photographic film is placed in contact with the solution to be tested. The enzyme digests away the gelatin from the surface of the film, and thereby removes some of the silver and increases the translucence of the film. The rate or degree of clearing thus effected is an accurate criterion of the gelatinase activity of the solution (2, 8).

The technic used in these experiments² differs only in several minor details from that used by Gates. The film was exposed as recommended by Gilman and Cowgill (8), developed with a hardener, dried, and again washed and dried in order to set the gelatin. The digestion cell was constructed with a flat brass ring instead

² Dr. William Mendelsohn rendered valuable assistance in the determination of proteolytic activity.

TABLE I
The Coagulant Action of Seventeen Snake Venoms

Coagulate citrated horse plasma		Do not coagulate citrated horse plasma	
Snake species	Common name	Snake species	Common name
<i>Bolhrops atrox</i>	Fer de lance	<i>Agkistrodon piscinarius</i>	Cotton-mouth moccasin
<i>Bolhrops jararaca</i>	Jararaca	<i>Bitis arietans</i>	Puff adder
<i>Bolhrops nummifera</i>	Mano de Piedra	<i>Crotalus atrox</i> *	Texas diamond back
<i>Crotalus adamanteus</i> †	Florida diamond back	<i>Crotalus horridus</i> *†	Timber rattler (1528) (1447)
<i>Crotalus horridus</i> †	Timber rattler (A29091A)	<i>Naja flava</i>	Cape cobra
<i>Crotalus terrificus basiliscus</i>	Cascabel, Mexican	<i>Naja naia</i>	Spectacled cobra
<i>Crotalus terrificus terrificus</i>	Cascabel, Brazilian	<i>Seledon haemachates</i>	Spitting cobra
<i>Micruis, mixed venoms</i>	Coral snake	<i>Vipera ammodytes</i>	Sand viper
<i>Notechis scutatus</i>	Australian tiger snake	<i>Vipera russellii</i>	Daboia

* 2 specimens.

† 3 specimens.

† It is to be particularly noted that in the case of *Crotalus horridus*, individual lots of venom presumably obtained from different members of the same species, nevertheless differed in their coagulating activity. This variation in different lots of venom may explain the fact that although Link (13) found the venom of *Crotalus adamanteus* actively coagulant, a finding which was confirmed in the present experiments, Houssey and Sordelli (9) report it inactive *in vitro*. Similarly, the venom of Russell's viper was here found inactive; Arthus (1) also reported it inactive; Link (13) obtained variable results; and Houssey and Sordelli (9), as well as Taylor, Mallick, and Ahuja (21) found it to be actively coagulant. *Crotalus terrificus* venom was here found to be regularly active in high dilution, confirming Houssey and Sordelli (9) and Arthus (1); but Link (13) found the venom or by the varying age of the preparations, or whether they reflect intrinsic differences in venoms obtained from different individuals of the same species, is an open question which need not concern us here.

of copper wire. The ring, 2 mm. thick, 25 mm. outside and 20 mm. inside diameter, was sealed with paraffin to a glass slide measuring 3.5 x 3.5 cm. The compartment so formed was filled with the solution to be tested, and a section of film 30 mm. square was placed on the solution, gelatin side down. Over the film was placed another glass slide, and the entire cell was then clamped together with two ordinary wooden spring-clamps, one on each side. The cell was then placed in a water bath at 28°C., the film exactly upright.

Instead of measuring the translucence of the washed film after a fixed interval with a colorimeter (8), complete clarification was used as a rough end-point of digestion (2). This end-point was made somewhat more accurate by observing the sediment of silver which gradually settled to the bottom of the cell as it was freed from the dissolved gelatin. The time at which this sediment formed a sharply demarcated layer which filled the lower $\frac{1}{4}$ of the cell, and left clear transparent film above, was taken as the end-point. The proteolytic activity of the several preparations was estimated by determining the maximum dilution of enzyme which produced this degree of clarification in an arbitrary time interval.

The Coagulation of Fibrinogen by Snake Venoms

The list of venoms tested, and their coagulative activity on plasma or on solutions containing both prothrombin and fibrinogen, are given in Table I. As is there shown, 8 of the 17 venoms tested proved active, and one (*Crotalus horridus*) yielded variable results. The discrepancies between the results there listed and the data in the literature are discussed in the footnote to Table I, and are not germane to the present paper.

In confirmation of previous reports, 7 of the 9 venoms which coagulated plasma were found capable of coagulating purified fibrinogen (Table II). These 7 venoms (*Bothrops atrox*, *Bothrops jararaca*, *Bothrops nummifera*, *Crotalus adamanteus*, *Crotalus terrificus terrificus*, *Crotalus terrificus basiliscus* and one of three specimens of *Crotalus horridus*) therefore contained a substance which, like thrombin or papain, reacted with fibrinogen to form a fibrillar gel indistinguishable from fibrin.

It is to be noted that this coagulative action is independent of the presence of calcium ions, as it occurs just as promptly in fibrinogen solutions containing 1 per cent sodium citrate. The coagulation is also independent of the presence of tissue or platelet derivatives, as it occurs in fibrinogen solutions which contain these factors only in minimal concentration. Moreover, the velocity of coagulation is

wholly unaffected by the addition of cephalin or tissue extracts to the fibrinogen. Finally, the coagulative action is independent of the presence of prothrombin, for fibrinogen which is prothrombin-free, and which is unaffected by $\text{Ca} + \text{cephalin}$, is nevertheless coagulated by the venoms. Clearly, the fibrinogen as such is attacked by the venoms directly.

An experiment to determine the optimum pH for this reaction is summarized in Fig. 1. As is there shown, each of the 3 venoms tested

TABLE II

*The Coagulation of Purified Fibrinogen by Snake Venoms**

To 0.4 cc. of a solution of purified horse fibrinogen were added 0.4 cc. of varying concentrations of snake venom. The figures in the body of the table indicate the coagulation time in minutes. Each horizontal row is an individual experiment.

Venom used	Concentration of venom added to an equal volume of purified fibrinogen							
	1:1000	1:2000	1:4000	1:8000	1:16,000	1:32,000	1:64,000	1:128,000
	min.	min.	min.	min.	min.	min.	min.	min.
<i>Bothrops atrox</i>	1½	1¾	2¼	2¾	4	5	8	12
<i>Bothrops jararaca</i>	1½	1½	2¼	2¾	4	6½	3	—
<i>Bothrops nummifera</i>	¾	¾	1¼	1¾	3¾	7	14	—
<i>Crotalus adamanteus</i> (1483)....	—	—	—	2	4½	10	15	—
(1336).....	½	¾	1	2	3½	5¾	11	18
(1458).....	½	¾	¾	1½	2	4	6¾	16
<i>Crotalus horridus</i> (A2091A).....	½	¾	1¼	1¾	2¾	4¼	7½	19
<i>Crotalus terrificus terrificus</i>	¾	¾	1¾	2½	3¾	6	8½	—
<i>Crotalus terrificus basiliscus</i>	2¼	2	3	5	10	21	37	—

* The following venoms caused no coagulation in 60 minutes: *Agkistrodon piscivorus*, *Crotalus atrox*, *Crotalus horridus* (lots 1528, 1447), *Micrurus mixed*, *Naia flava*, *Naia naia*, *Notechis scutatus*, *Sepedon haemachates*, *Vipera russellii*, *Bitis arietans*, *Vipera ammodytes*.

had a definite optimum at approximately pH 6.5; and it is to be noted that this optimum coincides with that for the action of thrombin on fibrinogen (Fig. 5, bottom curve). Unexpectedly, the coagulant action of snake venom on fibrinogen is unaffected by antithrombin. The intensely antithrombic plasma produced by anaphylactic shock in dogs (5 b) had no demonstrable effect on the coagulation of fibrinogen by *Crotalus adamanteus* or *Crotalus terrificus terrificus* (Table VI, page 629).

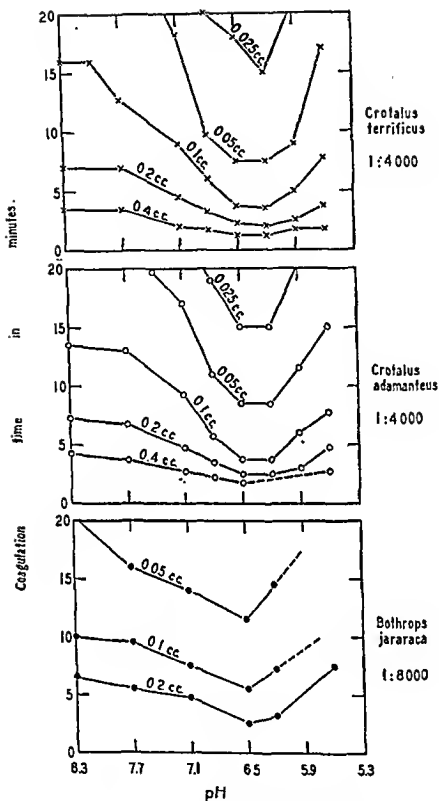


FIG. 1. The effect of pH on the fibrinogen-coagulating activity of several snake venoms.

Varying amounts of venom solution were made up to 0.4 cc. with 0.85 per cent NaCl solution, and 0.4 cc. of 0.1 M phosphate buffer was added to each tube. Finally, 0.4 cc. of unbuffered fibrinogen solution was added, and the coagulation time noted. The final experimental mixture therefore contained 0.033 M phosphate buffer and 0.105 M NaCl. In plotting the results, the pK' of this buffer mixture has been taken as 6.8. A stated pH of 7.1 represents a 2:1 Na_2HPO_4 ratio, a pH of 7.4 is a 4:1 mixture, etc. These pH values are in error to the extent that the pK' of these buffer solutions of varying ionic strength differs from 6.8, and the pH values are accordingly accurate only to ± 0.1 pH units.

Of the 11 venoms which did not coagulate fibrinogen, 5 (*Aghistrodon piscivorus*; *Bilis arietans*; *Crotalus atrox*; *Vipera ammodytes*; 2 of the

TABLE IV

The Correlation between the Proteolytic Activity of the Several Venoms as Measured with Gelatin, and Their Ability Either to Coagulate or Destroy Fibrinogen

Venom used	Proteolytic activity* of the dry venom as compared with crystalline trypsin 1:10,000		Coagulate fibrinogen	Destroy fibrinogen
	Experiment 1	Experiment 2		
<i>Aghistrodon piscivorus</i>		96		+ (16)†
<i>Bothrops nummifera</i>	50	64	+	
<i>Bothrops atrox</i>	36	70	+	
<i>Crotalus terrificus basiliscus</i>	16	40	+	
<i>Bothrops jararaca</i>	12	25	+	
<i>Crotalus atrox</i>	8	12		+ (8)
<i>Crotalus horridus</i> (2091A).....	5	8	+	
<i>Crotalus horridus</i> (1528).....	4	4		+ (4-8)
<i>Crotalus adamanteus</i>	4	5	+	
<i>Vipera ammodytes</i>	3	4		+ (4)
<i>Vipera russellii</i>	2	2	†	(3)
<i>Bilis arietans</i>	1½	1½		+ (2)
<i>Crotalus terrificus terrificus</i>	½	1½	+	
<i>Sepedon haemachates</i>	¾	1		
<i>Naia flava</i>	½	½		
<i>Micrurus mixed</i>	½	½		
<i>Naia naia</i>	¼	1		
<i>Notechis scutatus</i>	¼	¾		

* Cf. page 615 for the technic used in approximating the proteolytic activity of the various venoms.

† The numbers in this column indicate, very roughly, the relative fibrinogen-destroying activity of the several venoms as given in Table III. The differences in the coagulating activity (Table II) are too slight to justify quantitative analysis.

‡ In this list of 18 venoms, this constitutes the only exception to the observation that the capacity of the venom to coagulate or to destroy fibrinogen goes hand in hand with its proteolytic activity as measured with gelatin. It is to be noted that other observers have found this particular venom to be actively coagulant.

3 specimens of *Crotalus horridus*) were found to attack the protein, and render it non-coagulable by thrombin (Table III). The slight re-

tardation of coagulation caused by several of the remaining 6 venoms (Table III, lower half) is of questionable significance.

The question now arises as to whether the coagulative action of these snake venoms on fibrinogen is due to their proteolytic enzyme content. These venoms are heterogeneous mixtures of many different substances, and the demonstration of such enzymes does not prove them to be the factor responsible. Moreover, the actual demonstration of proteolysis in a fibrinogen-venom mixture is *a priori* difficult in view of: (a) the minute molecular concentration of protein in solution; (b) the possibility that only a few groups in the protein need be modified in order to produce a profound change in its biologic properties; and (c) the fact that one ends with a gel-like fibrinous mass which precludes precise formol titrations.

There is, nevertheless, a certain amount of collateral evidence that the coagulation of fibrinogen just described is due to proteolytic enzymes present in the venom. In the first place, the extraordinarily minute amounts of venom which may suffice is strongly suggestive. A 1:100,000 dilution of some of the crude venoms, in which the actual coagulant is only a small fraction of the total solid, regularly coagulated solutions containing 1:200 parts of fibrinogen, a minimum ratio of 500:1 between the two reagents.

Further evidence for the enzyme thesis is given by the data of Table IV. As is there shown, all 17 venoms used in these experiments contained proteolytic enzymes, capable of hydrolyzing gelatin. Their activity in this respect varied widely. The significant feature of Table IV is that only those venoms which contained comparatively large amounts of enzyme were able to coagulate or to destroy fibrinogen. If the particular samples of venom here used were arranged in the order of their gelatin-splitting activity, it was found that those venoms below a certain level of proteolytic activity had little or no effect on fibrinogen,³ while those venoms above this level either actively coagulated the protein, or rendered it non-coagulable even by thrombin. This almost exact correlation between the hydrolytic activity of the venoms

³ Within the time limits of the present experiments. It is not improbable that with a longer period of incubation, or at higher temperatures, even these weakly proteolytic venoms would have destroyed fibrinogen; but this possibility does not affect the validity of the argument.

as tested with gelatin on the one hand, and their ability either to destroy or to coagulate fibrinogen on the other, justifies the working hypothesis that the coagulation, like the destruction, is caused by one of the proteolytic enzymes present in the venom. One can only speculate as to why, given venoms of equal proteolytic activity, some hydrolyze fibrinogen to soluble products, while others hydrolyze it to an insoluble fibrillar gel indistinguishable from fibrin.

The Activation of Prothrombin to Thrombin by Snake Venoms

The venoms were now tested with respect to their ability to activate prothrombin to thrombin. Varying amounts of each venom were added to a fixed volume of purified prothrombin solution, and the coagulating activity of the mixture was tested after half an hour at room temperature (Table V). Of the 17 venoms tested, 3 were remarkably active (*Notechis scutatus*, *Bothrops atrox*, *Bothrops jararaca*), and regularly caused a definite transformation of prothrombin to thrombin when used in dilutions as high as 1:1,000,000 (the first 2 named were definitely active in 1:10,000,000 dilutions). Two other venoms (*Micrurus* mixed and *Crotalus terrificus basiliscus*) were weakly active.

Of the 5 venoms found capable of transforming prothrombin to thrombin, 2 (*Notechis scutatus* and a mixed *Micrurus* venom) had no effect on fibrinogen (Table II), and their coagulant action on whole plasma previously noted is apparently to be ascribed solely to the fact that they transform prothrombin to thrombin. The other 3 venoms which activated prothrombin acted also on fibrinogen. The activation of prothrombin in the case of these 3 venoms was demonstrated by the fact that a mixture of venom and prothrombin was 5, 50, or even 1000 times as active as the venom alone. For purposes of contrast, several venoms are included in Table V which actively coagulated fibrinogen but which did not activate prothrombin to thrombin.

That the coagulant produced in the venom-prothrombin mixtures was truly thrombin, and not the result of some wholly unrelated reaction, was indicated by the following observations.

1. The amount of coagulant formed with an optimum concentration of venom was of the same order of magnitude as that elab-

Mixed Micurus	0.4 0.1 0.025	1.2 1.2 1.2	1:4000 1:16,000 1:64,000	3 7 20	5 12 32	9 20 60	12 — —		"	"	"
<i>Crotalus terrificus</i> <i>basiliscus</i>	0.4	1.2	1:4000	11	2	31	7	21			2
	0.1	1.2	1:16,000	21	5	9	16	71			5
	0.025	1.2	1:64,000	91	18	35	60	36			4
<i>Crotalus adamanteus</i>	0.4	1.2	1:4000	11				1/4			Venom-prothrombin mixture uniformly less active than the venom alone. No formation of thrombin
	0.2	1.2	1:8000	2				11			
	0.1	1.2	1:16,000	31				2			
	0.05	1.2	1:32,000	41				4			
	0.025	1.2	1:64,000	91				71			
<i>Crotalus horridus</i>	0.4	1.2	1:4000	11				11			"
	0.2	1.2	1:8000	11				11			
	0.1	1.2	1:16,000	3				21			
	0.05	1.2	1:32,000	7				41			
	0.025	1.2	1:64,000	10				71			
<i>Crotalus terrificus</i> <i>terrificus</i>	0.4	1.2	1:4000	2				11			"
	0.2	1.2	1:8000	21				21			
	0.1	1.2	1:16,000	5				31			
	0.05	1.2	1:32,000	8				6			
	0.025	1.2	1:64,000	161				81			

* The coagulation time was used as the index of thrombin concentration (page 615). Thus, in the first horizontal row 0.2 cc. of venom was as active as 0.028 cc. of the venom-prothrombin mixture, a ratio of 0.2:0.028 = 7:1.

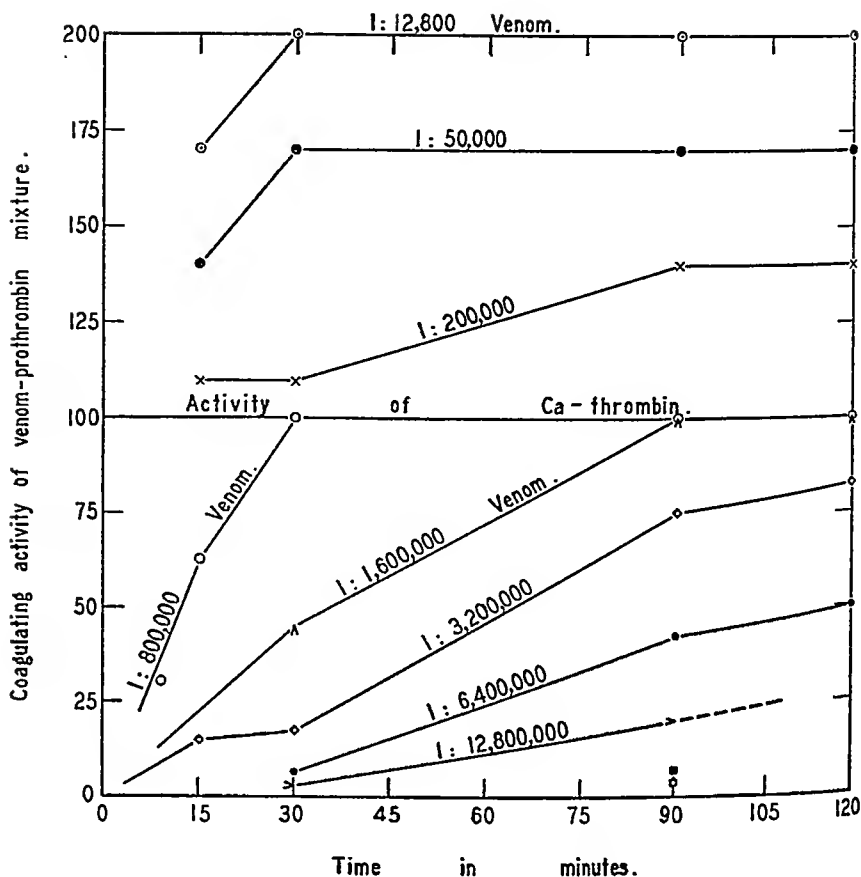


FIG. 2. The activation of prothrombin to thrombin by *Bolthrops atrox* venom.

Varying amounts of *Bolthrops atrox* venom were brought up to 0.4 cc. with $M/7$ NaCl, and 2.8 cc. of prothrombin solution were added. At intervals thereafter, aliquot samples were withdrawn and tested for thrombin content by adding varying amounts to 0.4 cc. of fibrinogen solution in a total volume of 0.8 cc. The coagulation time served as an index of thrombin content. The dilutions indicated on the curves are the final concentration of venom in the venom-prothrombin mixture.

The apparent production of thrombin in excess of the amount formed from the same prothrombin by calcium and tissue extract is due to the fact that the venom in concentrations exceeding 1:500,000 has a definite coagulant action on fibrinogen directly, independent of its action on prothrombin, and additive thereto.

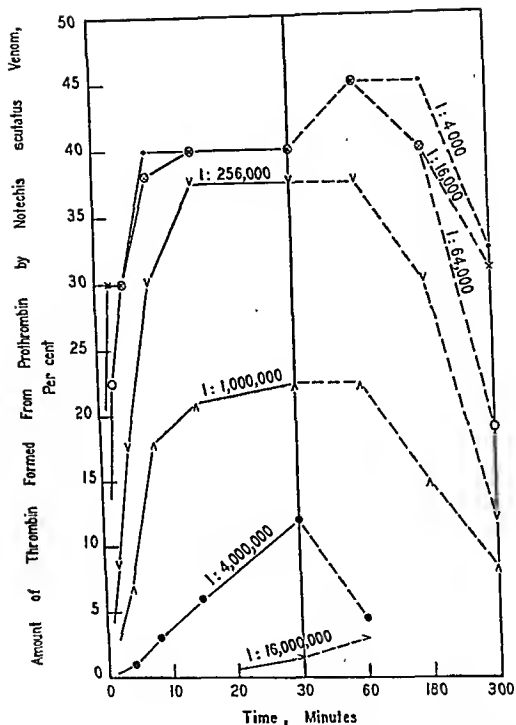


FIG. 3. The activation of prothrombin to thrombin by varying concentrations of *Notechis scutatus* venom.

Varying amounts of *Notechis scutatus* venom were brought up to 0.4 cc. with $\frac{1}{2}$ M NaCl, and 1.2 cc. of horse prothrombin solution (Mellanby purified) were added. At intervals thereafter, aliquot samples were withdrawn and tested for thrombin content by adding varying amounts to 0.4 cc. of fibrinogen solution in a total volume of 0.8 cc. The coagulation time served as an index of thrombin content. The dilutions indicated on the curves are the final concentration of venom in the venom-prothrombin mixture.

In plotting the thrombin activity, the maximum amount formed from the same prothrombin by calcium and cephalin was taken as 100.

TABLE VI

The Neutralizing Effect of Antithrombin on the Thrombin Produced by the Action of Bothrops jararaca or Bothrops atrox Venom on Prothrombin; and the Absence of Such Neutralization on Adding Antithrombin to Venoms Which, Like Thrombin, Act Directly on Fibrinogen (Protocol I)

Fibrinogen	Normal plasma	Post-anaphylaxis dog plasma (anti-thrombin)	Type of thrombin added	Coagulation time, on adding varying amounts of thrombin to the fibrinogen-plasma mixtures										Conclusion
				0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0062 cc.	0.0031 cc.			
	cc.	cc.		min.	min.	min.	min.	min.	min.	min.	min.			
A	0.4	—	<i>Bothrops atrox</i> + horse prothrombin	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{3}{4}$	$1\frac{1}{2}$	2	$3\frac{1}{2}$	6	Antithrombin markedly inhibits the thrombin formed from prothrombin by snake venoms, to the same degree that it inhibits thrombin formed from prothrombin by calcium + tissue extract		
	0.2	0.2		$\frac{1}{2}$	$\frac{1}{2}$	1	2	4	18	60				
	0.2	—		$3\frac{1}{2}$	7	19	60	No coagulation	No coagulation	No coagulation				
	0.4	—	<i>Bothrops jararaca</i> + dog prothrombin	$\frac{3}{4}$	$1\frac{1}{2}$	2	$3\frac{1}{2}$	6	12	20	35			
	0.2	0.2		$\frac{3}{4}$	$1\frac{1}{2}$	4	19	60	No coagulation after 4 hrs.	No coagulation after 4 hrs.				
	0.2	—		$4\frac{1}{2}$	29									
	0.4	—	Calcium + tissue extract + horse prothrombin	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{3}{4}$	1	2	$3\frac{1}{2}$	7	Antithrombin has no effect on snake venoms which act directly on fibrinogen to form fibrin		
	0.2	0.2		$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$1\frac{1}{2}$	$2\frac{1}{2}$	$4\frac{1}{2}$	25	No coag.			
	0.2	—		4	9	25	90	No coagulation	No coagulation	No coagulation				
	0.4	—	<i>Bothrops atrox</i> venom 1:4000	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	1	$1\frac{1}{2}$	$2\frac{1}{2}$	4	$6\frac{1}{2}$			
	0.2	0.2		$\frac{1}{2}$	$\frac{1}{2}$	$\frac{3}{4}$	$\frac{3}{4}$	$1\frac{1}{2}$	$2\frac{1}{2}$	4	7			
	0.2	—		$\frac{1}{2}$	$\frac{1}{2}$	$\frac{3}{4}$	$1\frac{1}{2}$	$2\frac{1}{2}$	$6\frac{1}{2}$	7	10			
B	0.4	—	<i>Bothrops jararaca</i> venom 1:4000	$\frac{1}{2}$	1	2	4	7	15	25	60	Antithrombin has no effect on snake venoms which act directly on fibrinogen to form fibrin		
	0.2	0.2		$\frac{1}{2}$	$1\frac{1}{2}$	2	$3\frac{1}{2}$	$6\frac{1}{2}$	15	29	60			
	0.2	—		$\frac{3}{4}$	$1\frac{1}{2}$	$2\frac{1}{2}$	$4\frac{1}{2}$	9	17	35	80			
	0.4	—	<i>Crotalus adamanteus</i> venom 1:4000	—	1	2	3	7	14	—	—			
	0.2	0.2		—	1	2	$3\frac{1}{2}$	8	12	—	—			
	0.2	—		$\frac{1}{2}$	$1\frac{1}{2}$	$2\frac{1}{2}$	$4\frac{1}{2}$	7	15	30	—			
	0.4	—	<i>Crotalus terrificus</i> venom 1:4000	$\frac{1}{2}$	$\frac{3}{4}$	$\frac{1}{2}$	$2\frac{1}{2}$	5	9	—	—			
	0.2	0.2		$\frac{1}{2}$	$\frac{3}{4}$	$1\frac{1}{2}$	$2\frac{1}{2}$	6	12	19	—			
	0.2	—		$\frac{1}{2}$	$\frac{3}{4}$	$1\frac{1}{2}$	$2\frac{1}{2}$	9	15	15	—			

orated from the same prothrombin by the addition of calcium and platelets (or tissue extract), (Figs. 2 and 3).

2. The addition of calcium and tissue extract to the optimum mixture of protbrombin and *Bothrops atrox* venom caused no further increase in its coagulating activity.⁴ Conversely, if venom was added to the thrombin produced by the action of calcium and platelets on prothrombin, there was no increase in coagulating activity unless the venom was added in sufficient concentration to supplement the thrombin by virtue of a direct action on fibrinogen (Fig. 2).

3. The antithrombin which is elaborated in dogs after anaphylactic shock (5b) is strictly specific for thrombin, and does not affect the coagulating activity either of papain or of those venoms which, like thrombin, act directly on fibrinogen. On the other hand, this antithrombin neutralized the coagulating activity of a prothrombin-venom mixture to exactly the same degree as it did a protbrombin-calcium-platelet mixture (Protocol 1 and Table VI).

Protocol 1

The neutralizing effect of antithrombin on the thrombin produced by *Bothrops jararaca* or *Bothrops atrox* venom acting on prothrombin, and the absence of such neutralization on adding antithrombin to venoms which, like thrombin, act directly on fibrinogen (Table VI, A and B).

The antithrombin used was citrated plasma obtained from dogs after anaphylactic shock, which contained approximately 25 times the normal amount of antithrombin (5 b). *Jararaca* thrombin was prepared by adding 0.4 cc. of a 1:10,000 solution of the venom to 4 cc. of horse or dog purified prothrombin. *Arox* thrombin was prepared similarly, using a 1:50,000 solution of the venom. The final concentrations of venom (1:100,000 and 1:500,000, respectively) had no demonstrable effect on fibrinogen within the time limits of the present experiment. Physiologic thrombin was formed by adding 0.4 cc. of a 1 per cent CaCl_2 and a trace of lung extract to 4 cc. of prothrombin solution. The calcium in the physiologic thrombin was rendered inactive by the addition of $\frac{1}{10}$ volume of 3 per cent citrate just before its use in the experiment.

After 1 hour at room temperature, varying quantities of each thrombin were brought up to 0.4 cc. and added to: (a) 0.4 cc. fibrinogen; (b) 0.2 cc. fibrinogen +

⁴ In the case of *Notechis scutatus*, even at the optimum prothrombin:venom ratio, only half of the prothrombin was converted to thrombin (Fig. 3); and on the subsequent addition of calcium and cephalin, the remainder of the prothrombin was converted.

0.2 cc. normal dog plasma; (c) 0.2 cc. fibrinogen + 0.2 cc. post-anaphylaxis dog plasma, and the coagulation time noted (Table VI, section A).

To test the effect of antithrombin on those venoms which act directly on fibrinogen, varying amounts of venom were brought up to 0.4 cc., and added to: (a) 0.4 cc. fibrinogen; (b) 0.2 cc. fibrinogen + 0.2 cc. normal plasma; (c) 0.2 cc. fibrinogen + 0.2 cc. post-anaphylaxis dog plasma. The results are given in Table VI, section B.

As is there shown, antithrombin did not affect the direct action of venoms on fibrinogen, but it did markedly inhibit the thrombins formed by the interaction of venom and prothrombin.

These several observations indicate that the venoms of *Bothrops atrox*, *Bothrops jararaca*, *Notechis scutatus*, and to a slight degree, *Crotalus terrificus basiliscus* and a mixed *Micrurus* venom, can transform prothrombin to thrombin. Indeed, despite the fact that these venoms are a heterogeneous mixture of substances, the first 3 named are many times as effective in this respect as crystalline trypsin.

As in the case of trypsin, the activation of prothrombin by snake venoms occurs in the absence of ionized calcium and in the absence of any demonstrable platelet or tissue derivative (cephalin). Strongly citrated plasma was coagulated by *Notechis scutatus* venom, which had no effect on fibrinogen; and prothrombin containing as high as 1 per cent citrate was promptly converted to thrombin by any of the 5 active venoms. Similarly, prothrombin which contained no demonstrable tissue or platelet derivative, and which was unaffected by the addition of calcium alone, was nevertheless activable by these venoms. Moreover, the addition of cephalin, platelets, or tissue extracts had no significant effect, either on the rate of thrombin production, or the amount ultimately formed. It seems clear that the venoms acted on prothrombin directly, and that neither calcium nor cell derivative was necessary for the observed production of thrombin.

Unlike trypsin, these 5 venoms did not rapidly digest the formed thrombin under the conditions of the experiment. In consequence, there was no optimum concentration of venom for the activation of prothrombin, but a broad zone over which the maximum amount of thrombin produced was more or less constant. A minute amount of venom sufficed to cause a maximum production of thrombin, and even a hundredfold excess over and above this necessary minimum had no demonstrable effect other than to increase the rate of thrombin

production (Figs. 2 and 3). It is to be particularly noted that in the physiological coagulation of blood there is a similar relationship between the amount of the activating principle (platelet derivative) and the rate and degree of thrombin production (3).

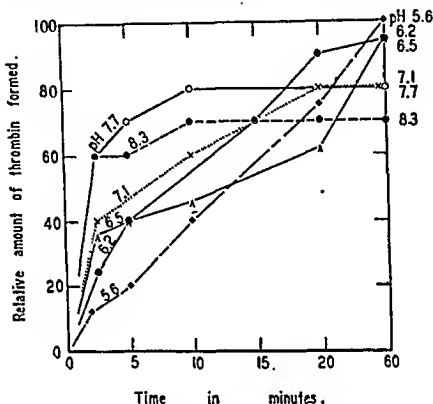


FIG. 4. The effect of pH on the rate and degree of thrombin production from prothrombin by *Bothrops atrox* venom.

To 0.8 cc. of purified prothrombin solution were added 0.8 cc. $M/7$ NaCl, 0.4 cc. $M/10$ phosphate buffer, and 0.4 cc. of a 1:50,000 dilution of *Bothrops atrox* venom. At the intervals indicated on the figure, varying samples were withdrawn and added to 0.4 cc. of fibrinogen and 0.1 cc. of the same phosphate buffer in a total volume of 0.8 cc.

The coagulation time served as the index of thrombin activity. It is to be noted that the reference curves on which these times were interpolated vary with the pH. Accordingly, a reference curve had to be constructed for each pH, based on data similar to those of Fig. 5.

The pH values given in Fig. 4 are based on a pK' of 6.8 for the various buffer mixtures, and are in error to the extent that this pK' deviates from 6.8 in $M/60$ phosphate buffer mixtures of varying ionic strength (cf. legend to Fig. 1).

One of three experiments to ascertain the effect of pH on the rate and degree of thrombin formation from prothrombin by *Bothrops atrox* venom is summarized in Fig. 4. As is there shown, over the pH range 5.6 to 8.3, increasing alkalinity caused a progressive increase in the

COAGULATION OF BLOOD BY SNAKE VENOMS

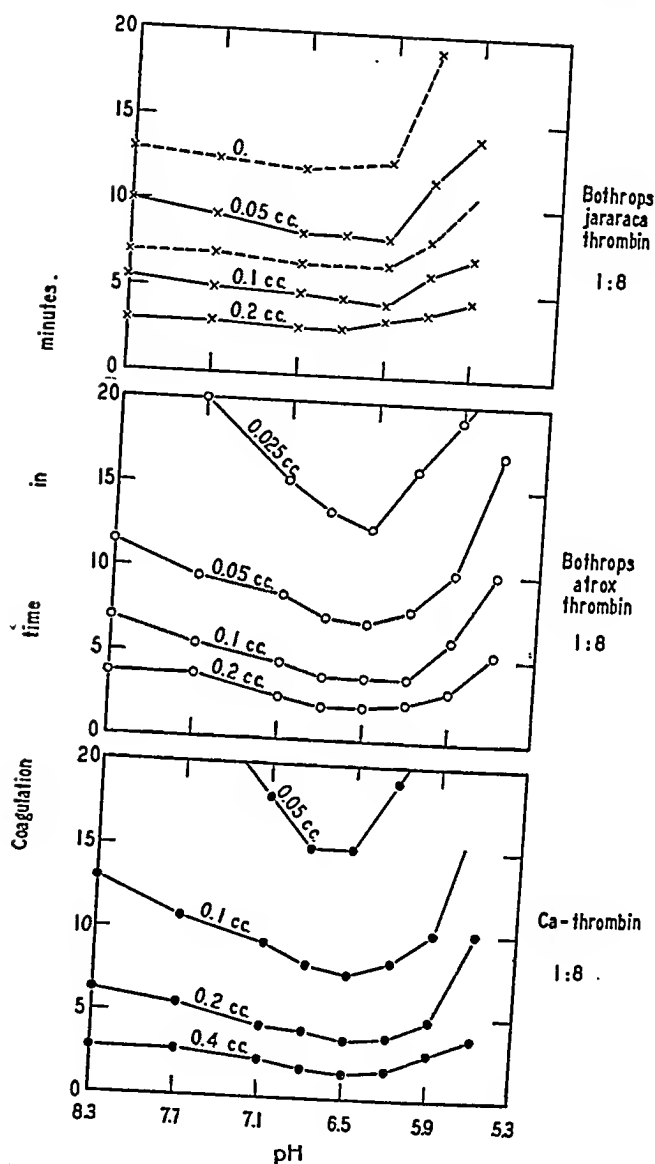


FIG. 5. The optimum pH for the action of various types of thrombin on fibrinogen.

To 0.8 cc. of purified horse prothrombin solution were added 1.2 cc. of $M/7$ NaCl and either (a) 0.4 cc. of 1:20,000 *Bothrops atrox* venom, (b) 0.4 cc. of a 1:8000 *Bothrops jararaca* venom, or (c) 0.2 cc. of 1 per cent $CaCl_2$ + 0.1 cc. of lung extract and 0.1 cc. $N/7$ NaCl. None of the solutions were buffered. After 1 hour

initial rate of thrombin production, but the amount ultimately formed steadily decreased. The difference between adjoining curves is no greater than the experimental error, but the consistency of the trend strongly suggests that these are real differences. The observation is as yet unexplained.

It is perhaps significant that the optimum pH for the coagulative action of the thrombin formed from prothrombin by *Bothrops atrox* or *Bothrops jararaca* venom, pH 6.5 (Fig. 5), coincides with that for the action of thrombin as formed from prothrombin physiologically by calcium and platelets (23), and coincides also with the optimum pH for the coagulative action of various venoms which act on fibrinogen directly (Fig. 1).

The question arises as to whether the conversion of prothrombin to thrombin by the 5 venoms found to be active in this respect can be ascribed to the proteolytic enzymes present in these venoms. The quantitative disproportion between the prothrombin and venom is of interest in this connection. A 1:2,000,000 dilution of *Bothrops atrox* often produced a complete activation of prothrombin to thrombin; and 1:25,000,000 dilutions had a definite, if partial, effect (Fig. 2; Table V). Since the protein concentration in the prothrombin solutions was approximately 0.05 per cent, the reacting proportions therefore varied between 1:1000 and 1:10,000. Granted that both the venom and the prothrombin solutions are crude preparations in which the active principle probably constitutes only a small and as yet indeterminate proportion of the total solid, this disproportion strongly suggests some reaction other than simple chemical combination.

In the second place, prothrombin is always associated with the

at room temperature, varying amounts of each type of thrombin were added to 0.4 cc. of fibrinogen solution + 0.2 cc. M/10 phosphate buffer in a total volume of 1 cc., and the coagulation time noted.

The curves in the top section of the figure (*Bothrops jararaca* thrombin) represent two experiments with different prothrombin and fibrinogen preparations. The experiment summarized in the lower section of the figure (calcium-thrombin) was carried out with yet another batch of reagents. The absolute activities of the several thrombins are therefore not comparable. As is evident in the figure, the optimum pH is more clearly shown with the smaller amounts of thrombin, and centers at pH 6.3 to 6.7.

globulin fraction of plasma, and its known properties are wholly consistent with the thesis that it is itself a protein. It is destroyed by known proteolytic enzymes, such as trypsin and papain (5a), and it was similarly digested by 9 out of the present series of 17 venoms (Table VII), including some only weakly proteolytic (Table IV). It is accordingly difficult to understand why the 5 venoms which did activate prothrombin to thrombin and which include some of the most

TABLE VII
*The Destruction of Prothrombin by Snake Venoms**

Venom used	Amount of 0.1 per cent solution added to 0.4 cc. prothrombin solution								Conclusion
	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0062 cc.	0	
	Coagulation time on adding CaCl ₂ , tissue extract, and, ½ hour later, 1.6 cc. fibrinogen in a total volume of 3.2 cc.								
	min.	min.	min.	min.	min.	min.	min.	min.	
<i>Agkistrodon piscivorus</i> ...	∞†	∞	∞	∞	50	4	2¼	½	All these venoms actively destroyed the prothrombin, and rendered it non-activable by calcium + tissue extract
<i>Bilis arietans</i>	∞	∞	∞	10	—	—	—	½	
<i>Crotalus atrox</i> (1446).....	∞	∞	∞	4	—	—	—	½	
(67248).....	∞	∞	∞	∞	∞	∞	2	½	
<i>Crotalus horridus</i> (1447)...	∞	∞	∞	∞	∞	∞	6	½	
(1528)...	∞	∞	∞	∞	∞	5	—	½	
<i>Naia flava</i>	90	5	5	4½	4	2½	1¾	¼	
<i>Naia naia</i>	∞	∞	5	2½	1	¾	¾	½	
<i>Sepedon haemachates</i>	∞	∞	∞	4	—	—	—	½	
<i>Vipera ammodytes</i>	∞	∞	13	1½	¾	¾	¾	¾	
<i>Vipera russellii</i>	—	∞	12	2¼	1¾	¾	¾	¾	

* *Crotalus adamanteus*, *Crotalus terrificus terrificus*, and *Bothrops nummifera* could not be tested because the venom *per se* coagulated fibrinogen.

† No coagulation in 2 hours, i.e., no significant production of thrombin.

actively proteolytic of this series (*Bothrops atrox*, *Bothrops jararaca*, *Crotalus basiliscus*), failed to digest the prothrombin, unless the proteolytic enzyme (or enzymes) was itself the cause of the transformation.

No explanation can be offered for the fact that some proteolytic venoms destroyed prothrombin, while others activated it to thrombin. In the analogous case of fibrinogen, it seemed fairly clear that the protein was affected only by venoms containing more than a certain

limiting concentration of proteolytic enzyme. Of those venoms which contained more than this necessary minimum, some digested the protein, and some coagulated it to form a fibrillar gel resembling fibrin. There was no correlation between the proteolytic activity as measured with gelatin and the type of effect produced on fibrinogen (Table IV). It was accordingly found necessary to assume that there were two types of venom enzymes as regards their effect on fibrinogen. In the case of prothrombin also, all these venoms affected the substance, but there was no apparent correlation between the magnitude of the proteolytic activity and the type of effect produced. The 5 venoms which activated prothrombin to thrombin include several of the most actively proteolytic (*Bothrops atrox*, *Bothrops jararaca*) and one of the least proteolytic (*Notechis scutatus*) of the present series. In this case also, we must assume that with respect to their effect on prothrombin there are two types of venom enzyme: one type which digests the substance and renders it wholly inactive, and a second type which activates it to thrombin.

An analogy is to be seen in the difference between trypsin and papain. In proper concentration, the former activates prothrombin and destroys fibrinogen, while the latter has exactly the opposite effect, *i.e.*, it destroys prothrombin and coagulates fibrinogen (5).

DISCUSSION

The present observations constitute additional evidence that the process of physiological coagulation involves two consecutive enzyme reactions.

Morawitz (19) originally suggested that platelets or tissue derivative constituted a kinase which, in the presence of calcium, activated prothrombin to thrombin. Although most subsequent workers in the field have not accepted this enzyme theory, it is nevertheless consistent with most of the known properties of the reaction (literature summarized in reference 4). It finds strong support also in the recent finding that crystalline trypsin may replace calcium and platelets, and is alone capable of transforming prothrombin to thrombin (5a).

As here reported, 5 of the 9 snake venoms found to coagulate plasma caused the conversion of prothrombin to thrombin. At least 9 of the remaining 12 non-coagulant venoms actively destroyed prothrombin.

For the reasons cited in the text, it is probable that the activation, like the destruction, is caused by proteolytic enzymes demonstrable in the venoms. It is particularly noteworthy that this activation proceeds in the absence of ionized calcium, and in the absence of cephalin, platelets, or tissue derivatives. As in the case of crystalline trypsin, these venoms therefore replace the physiological system calcium + platelet (or tissue) derivative in converting prothrombin to thrombin.

Given the observation that at least three systems may effect this transformation; that one of these systems (trypsin) is a crystalline proteolytic enzyme; and that a second system (snake venom) is a heterogeneous mixture of substances in which the active constituent is probably a proteolytic enzyme; it becomes an increasingly plausible working hypothesis that the third system, calcium and platelet (or tissue) derivative, also constitutes a proteolytic enzyme which activates prothrombin.⁵ It is an open question whether this hypothetical enzyme hydrolyzes or combines with its substrate, prothrombin, to form the actual coagulant, thrombin.

Schmidt (20), one of the earliest of the modern workers on the coagulation problem, termed this thrombin a fibrin ferment, and believed it to be a proteolytic enzyme which split fibrinogen to form fibrin. Although most recent workers have, for reasons which are of debatable validity (4), discarded the enzyme theory of thrombin, the finding (5) that a proteolytic enzyme, papain, acts directly on fibrinogen to form a fibrillar gel resembling that produced by thrombin, constitutes cogent evidence in that direction.

In the present experiments 7 of 9 coagulant venoms were found to act directly on fibrinogen to form a fibrillar gel, and 5 of 9 non-coagulant venoms actively destroyed the protein. Most significant, the ability of the venoms either to digest or to coagulate fibrinogen was directly related to their proteolytic activity as tested with gelatin. It was therefore concluded that the coagulant and destructive action of these venoms on fibrinogen was in all probability due to their proteolytic enzyme content.

In view of the observations (*a*) that papain, a proteolytic enzyme, converts fibrinogen to a fibrillar gel, and (*b*) that the similar action of

⁵ The one observation which seems difficult to reconcile with this hypothesis is the fact that a lipoidal tissue extract (cephalin?) can replace the tissue extractive.

certain snake venoms is probably due to their proteolytic enzyme content, it seems a valid working hypothesis that the physiological coagulant, thrombin, is also a proteolytic enzyme with a specific substrate, fibrinogen.

It is a well known observation that numerous bacteria, notably staphylococci, may cause the coagulation of decalcified blood or plasma. Since most bacteria elaborate proteolytic enzymes, one may well inquire whether this coagulative action may not be due to these enzymes, acting either on prothrombin to form thrombin, or directly on fibrinogen to form fibrin. Experiments in this direction are now in progress.

We may therefore tentatively view the coagulation phenomenon as the result of two consecutive enzyme reactions.

Physiological substrate		Activating enzyme	Product
1. Prothrombin	+	Calcium + platelets (tissue derivative; cephalin?) Trypsin Proteolytic snake venoms ⁶ Bacterial proteases?	Thrombin
2. Fibrinogen	+	Thrombin Papain Proteolytic snake venoms ⁷ Bacterial proteases?	Fibrin

As previously stated, this working hypothesis finds a complete analogy in the activation of chymotrypsinogen by a proteolytic enzyme, trypsin, to form a second enzyme, chymotrypsin (11).

SUMMARY

Nine of the 17 venoms here tested were found capable of coagulating citrated blood or plasma. As has been believed by most workers in the field, 7 of these 9 coagulant venoms convert fibrinogen to an

⁶ *Notechis scutatus*, *Bothrops atrox*, *Bothrops jararaca*, *Crotalus basiliscus*, a mixed *Micrurus* venom: there are undoubtedly others not included in the present series.

⁷ *Bothrops atrox*, *Bothrops jararaca*, *Bothrops nummifera*, *Crotalus adamanteus*, *Crotalus horridus*, *Crotalus terrificus basiliscus*, *Crotalus terrificus terrificus*: there are undoubtedly others not included in the present series.

insoluble modification resembling fibrin (*Bothrops atrox*, *Bothrops jararaca*, *Bothrops nummifera*, *Crotalus adamanteus*, *Crotalus horridus*, *Crotalus terrificus basiliscus*, *Crotalus terrificus terrificus*). The optimum pH for this coagulation was determined for 3 of these, and was found in each case to be approximately pH 6.5, the same as that for the action of thrombin on fibrinogen. Unlike thrombin, however, the fibrinogen-coagulating activity of the venoms was unaffected by the antithrombin elaborated in the course of anaphylactic shock.

In addition to coagulating fibrinogen directly, 3 of these venoms (*Bothrops atrox*, *Bothrops jararaca*, and to a less extent, *Crotalus terrificus basiliscus*) acted on prothrombin to convert it to thrombin, without the necessary intervention of either calcium or platelets. Finally, 2 venoms (*Notechis scutatus*, and to a slight extent, a mixed *Micrurus* venom), which had no demonstrable effect on purified fibrinogen, nevertheless converted prothrombin to thrombin.

Unlike the reaction between the venoms and fibrinogen, this activation of prothrombin has no definite pH optimum, but takes place over a wide zone (pH 5.6–8.3). In the case of *Bothrops atrox*, there was some indication that the initial velocity of the reaction increased with increasing alkalinity, but that the amount of thrombin ultimately formed decreased. Extraordinarily minute quantities of some of these venoms sufficed to produce a demonstrable activation of prothrombin. Thus, the fer de lance (*Bothrops atrox*) venom was active in a 1:25,000,000 dilution, and that of the Australian tiger snake (*Notechis scutatus*) was active in a 1:4,000,000 dilution.

The thrombin formed was indistinguishable from that produced by the action of calcium + platelets on prothrombin. Like the latter type of thrombin, and unlike venoms which act directly on fibrinogen, thrombin formed from prothrombin by venom was inhibited by anti-thrombin.

Every one of the 9 non-coagulant venoms in this series destroyed prothrombin; and 5 of these destroyed fibrinogen as well. As is discussed in the text, there is reason to believe that these several properties of the venoms (coagulation and destruction of fibrinogen; activation and destruction of prothrombin) depend on the proteolytic enzymes which they were found to contain.

These observations lend further support to the thesis that, in the

course of physiological coagulation, (a) calcium plus platelets (or tissue derivative) constitute an enzyme system which reacts with prothrombin to form thrombin, and which is thus analogous to trypsin and to several of the proteolytic venoms here discussed, and (b) the thrombin so formed is itself a proteolytic enzyme which, like papain and the majority of the coagulant and proteolytic snake venoms here studied, reacts with fibrinogen to form a fibrillar gel, fibrin.

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ELECTROPHORESIS OF PURIFIED ANTIBODY PREPARATIONS

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In connection with a study of the ultracentrifugal sedimentation of antibody preparations (1) the opportunity arose for study of the electrophoretic properties of some of the highly active material under investigation. Measurements of this kind are of value in the characterization of proteins and similar high molecular substances, and may also give information regarding the chemical homogeneity of the material, as shown in previous publications (2a).

The measurements given in the present paper form part of a more detailed, as yet uncompleted investigation of the electrochemical properties of proteins in normal and immune sera. Since, however, the ultracentrifugal study referred to above was made on the same material, it was thought advisable to publish the electrophoretic data at the present time.

Of the material used in the work of Heidelberger and Pedersen the following was investigated.

From Horse Sera.—Preparation 1: Obtained by dissociation of a Type I antipneumococcus specific precipitate with 15 per cent sodium chloride; 61 per cent of the total nitrogen was specifically precipitable ((1), Experiment 19).

Preparation 2: Obtained by the same dissociation method applied to the specific precipitate from a Felton solution from Type I antipneumococcus serum; 87 per cent of the nitrogen was specifically precipitable. This corresponds to the solution used in (1), Experiment 21.

Preparation 3: Dissociated Type I pneumococcus anticarbohydrate, obtained from unpreserved serum ((1), Experiment 18). More than 51 per cent of the nitrogen present was specifically precipitable.

From Rabbit Serum.—Preparation 4: Dissociated Type III. pneumococcus anticarbohydrate. 90 per cent of the nitrogen content was specifically precipitable ((1), Experiments 7 and 8).

The method for the electrophoresis measurements (ultraviolet photography of the moving boundaries) has been described in previous publications (2a, b). A

rather low potential gradient must be used on account of the high conductivity of the electrolyte media, which, for investigation of serum globulin must usually have a rather high concentration to keep the substance in solution. Preparation 4 furnishes an exception, being soluble in distilled water. Thus an ionic strength of $\mu = 0.1$ was used in all buffer solutions (acetate and phosphate) except those for the last mentioned preparation, for which $\mu = 0.02$ was used. The quantities available were rather small, usually sufficient for only one determination. However, it was possible to recover most of the material at the conclusion of each experiment by applying gentle suction to the capillary tube at the bottom of the U tube. The recovered solution was then dialyzed against the buffer to be used in the next experiment. The protein concentration was 0.2 per cent.

TABLE I

Electrophoretic Mobilities of Antibody Preparations at 20.0°C. in Acetate and Phosphate Buffer Solutions of Varying pH and Constant Ionic Strength

Buffer	pH	Mobility in cm. ² volt ⁻¹ sec. ⁻¹ $\times 10^5$			
		Preparation 1	Preparation 2	Preparation 3	Preparation 4
Acetate	4.63	+3.9	+4.3	+2.0	+6.7
Phosphate	5.24			-0.7	
"	5.30	+1.8		-1.5	
"	5.47				+5.7
"	6.23	-0.9		-3.3	
"	6.96				-1.5
"	7.34	-2.8	-3.5	-5.0	-2.8
Isoelectric point		pH = 5.94		pH = 4.9	pH = 6.7
Slope of curve at isoelectric point:		$du/dpH = 2.7 \times 10^{-5}$		3.5×10^{-5}	3.9×10^{-5}

The results are collected in Table I, and in Figs. 1 and 2. For comparison the mobility-pH curves for normal serum globulin from horse and rabbit sera (which are practically identical) have been plotted as broken lines in the corresponding diagrams (3).

Only preparation 3, which had been obtained from a serum containing no preservative, showed homogeneous electrophoresis. This material was also quite homogeneous in the centrifuge. However, too much stress should not be laid upon this result as far as electrophoresis is concerned. The voltage in these experiments is very low compared with that used in other cases (water soluble proteins) and some inhomogeneity might have escaped detection. It is therefore remarkable that the other three preparations showed a very pro-

nounced inhomogeneous migration. Such is also the case for serum globulin, as has been shown (3). While no indication of definite components could be observed in serum globulin, the antibody preparations 1 and 2 and possibly also 4 showed the presence of appreciable amounts of a substance migrating at a rate quite different from that of the bulk of the material. Its mobility could be calculated at pH 5.30 and pH 7.34, as -1.3×10^{-5} and -6×10^{-5} cm.² volt⁻¹ sec.⁻¹,

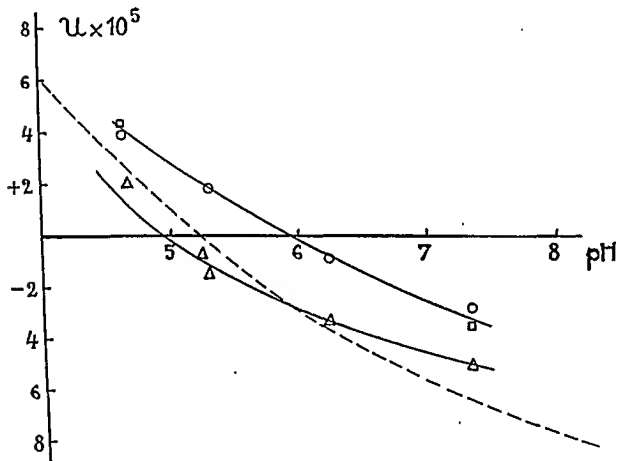


FIG. 1. Mobility of antibody preparations from horse serum at different pH (temperature 20.0°C.). O, preparation 1; □, preparation 2, Δ, preparation 3. Broken curve, normal horse serum globulin.

respectively. This component therefore has a much more acid isoelectric point, and it is to be noted that the mobilities found would fit fairly well into the curve for preparation 3, the homogeneous antibody from horse serum.

The rabbit serum preparation 4 gave much more diffuse boundaries on account of its higher diffusion, and did not allow similar calculations to be made with any degree of certainty.

If the acid component in preparations 1 and 2 is really identical with the homogeneous preparation 3, the bulk of the material in the inhomogeneous preparations would probably be denatured antibody. These solutions were also found to be very inhomogeneous in the ultracentrifuge. Pedersen has found that denaturation may be accompanied by a considerable shift in the isoelectric point toward the alkaline side (4). It would, however, still be necessary to account

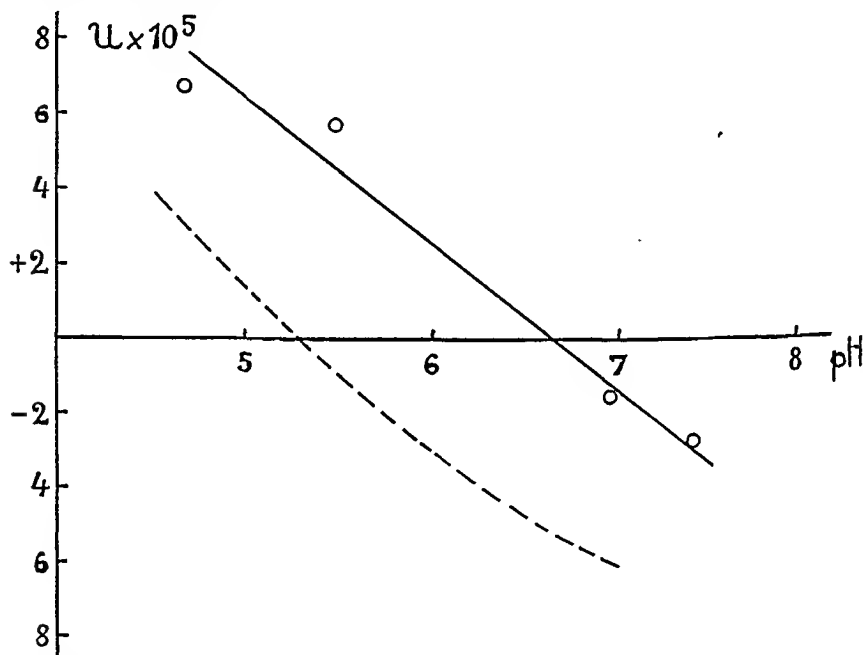


FIG. 2. Mobility of an antibody preparation (No. 4) from rabbit serum at different pH (temperature 20.0°C.). Broken curve, normal rabbit serum globulin.

for the finding that of the two last mentioned preparations No. 2 has a higher specific precipitability and No. 1 about the same as that of the homogeneous preparation 3.

At any rate the results as shown in Figs. 1 and 2 demonstrate that the antibody preparations are distinctly different from whole serum globulin. For all three horse serum preparations the low mobility at alkaline pH is characteristic. Evidently the number of ionized groups is unusually small. The rabbit antibody preparation differs

strikingly from normal rabbit serum globulin in its isoelectric point, which is especially noteworthy as these substances could not be distinguished in the ultracentrifuge. We have here one of the numerous instances of identical sedimentation but different electrochemical properties.

Of course, one may raise the objection that the observed differences between antibody and normal serum globulin are due to changes taking place in the course of preparation, although very mild methods have been applied. However, in some recent experiments with an improved apparatus (5) we have been able to show that normal and immune serum, which have been exposed only to dialysis against a buffer solution, show the presence of several differently migrating globulins when subjected to electrophoresis at high voltage. In immune sera the antibody function is found only in one of these fractions, namely one of the most slowly migrating components, in agreement with the results obtained above. A considerable purification of antibodies could be obtained in this way in horse as well as in rabbit sera (6).

SUMMARY

Electrophoretic mobilities of antibody preparations isolated from type specific antipneumococcus horse and rabbit sera, measured over a range of pH values, show that these preparations are distinctly different from normal serum proteins in their electrochemical properties.

We are much indebted to Dr. Michael Heidelberger for supplying the antibody preparations and for valuable suggestions.

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A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

IV. THE REACTION OF PNEUMOCOCCUS SPECIFIC POLYSACCHARIDES WITH HOMOLOGOUS RABBIT ANTISERA*

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It has been shown that the precipitin reaction between the specific polysaccharide of Type III pneumococcus and homologous antibody produced in the horse takes place according to the equation

$$\text{mg. antibody N precipitated} = 2RS - \frac{R^2}{A} S^2 \dots\dots\dots [1]$$

in which S is the amount of specific polysaccharide used and R is the ratio of the components in the precipitate at a reference point in the equivalence zone (1). It was found that this equation could be derived from the mass law if the reaction were considered the resultant of competing bimolecular reactions, and that in the derivation the volume factors cancelled, in agreement with the experimental observation that the composition and amount of the precipitate depended on the relative proportions in which the components were mixed, rather than on their concentrations. The formation of the precipitate was considered due to the building up of large aggregates by the union, according to the equation, of multivalent S with multivalent antibody.

It was also shown that this quantitative theory of the precipitin reaction was applicable to antigen-antibody systems (2, 3) as well as to the hapten-antibody reaction. Since differences had been observed between the behavior of antibodies produced in the horse and those

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derived from the rabbit toward specific polysaccharides and their degradation products (4), a study was initiated several years ago of the reaction between S III¹ and homologous antibody produced in the rabbit. However, only recent data are included in the present report, since it was found that pneumococcus specific polysaccharides were not thermostable, as had been supposed, and those used in the earlier work failed to yield maximal precipitates with rabbit antibody (5).

EXPERIMENTAL

Antisera.—Rabbits were injected intravenously with Type III pneumococci in the mucoid phase, strain A 66, killed by the addition of 2 per cent by volume of 40 per cent formalin solution. The suspension injected contained 0.05 mg. of bacterial nitrogen per ml. The immunizing dose was increased from 1 ml. to 3 ml. during a course of 15 injections, of which 3 to 4 were given per week. The initial bleedings, 3.50₁ and 3.51₁, were made 4 days after the last injection. After a rest period of 1 week a second course of 12 injections was given, bleedings 3.50₂ and 3.51₂ being taken 7 days after the last injection. Third and fourth courses of injections were given similarly. In these two rabbits the sera from the first course contained the largest amounts of antibody.

Rabbit 3.70 was given one course of acid-killed pneumococcus Type I suspension, strain I 230, but did not yield a potent antiserum until after nearly 40 additional injections of formalinized suspension.

Before use the sera were precipitated with C substance (6) in excess, in order to remove antibody which would react with traces of this substance remaining in the polysaccharide used.

Specific Polysaccharides of Types I and III Pneumococcus.—These substances were prepared without the use of heat, alkali, or strong acid, according to (5), since earlier methods of preparation yielded products with impaired ability to precipitate rabbit antisera.

*Determination of the Antibody Nitrogen Precipitated by Various Amounts of S I and S III.*¹—An accurately measured quantity of antiserum containing between 0.65 and 1.25 mg. of antibody nitrogen was added to various amounts of polysaccharide in saline at 0°, at a final volume of 4 ml. After 48 hours in the ice box the precipitates were centrifuged, washed with saline, and analyzed for nitrogen by the micro Kjeldahl method as described in earlier papers (7, 8). All supernatants were tested for both components by the addition of antiserum and polysaccharide to separate portions. Polysaccharide was estimated quantitatively in many of the supernatants according to (9).

¹ S I and S III are used throughout to designate the specific polysaccharides of Types I and III pneumococcus.

In Table I are given data on the solubility of the S III-anti-S III precipitate and the effect of temperature on the amount of antibody nitrogen precipitated. In Table II are given the amount of antiserum used, the quantity of antibody N precipitated by the given amounts of S III, the amount of nitrogen calculated from the equation for the reaction, the ratio between antibody N and S III in the precipitate, the tests on the supernatants, and notes on the appearance of the precipitates. Corresponding data for S I and serum 3.70 are given in Table V.

TABLE I

Effect of Temperature and Dilution on Antibody Precipitated from Type III Antipneumococcus Rabbit Sera

Antiserum	Volume used	Amount of S III	Final volume	Antibody N precipitated		Tests on supernatants
				at 37°	at 0°	
	ml.	mg.	ml.	mg.	mg.	
3.49 ₁ (1:1)	1.0	0.10	2.0	0.50	0.61	Excess S
3.51 ₁ (1:1)	1.0	0.060	2.0	0.50	0.55	Excess A
	1.0	0.079	2.0	0.55	0.61	Excess S
						Difference per ml.
						mg.
3.49 ₁ (1:1)	1.0	0.10	2.0		0.550*	0.007
	1.0	0.10	10.0		0.494*	
3.50 ₁ (2:1)	1.0	0.15	2.0		0.796*	0.006
	1.0	0.15	12.0		0.736*	
3.51 ₁ (1:1)	1.0	0.06	2.0		0.496*	0.003†
	1.0	0.06	9.0		0.474*	

* Run in triplicate. The supernatants were also centrifuged and any additional traces of precipitate were washed with the washings from the main portions.

† In this series the larger tubes were washed with 3.5 ml. more saline than were the smaller tubes. Correcting for this, the difference per ml. is reduced to 0.002 mg.

DISCUSSION

Although antisera produced in the rabbit were included in earlier quantitative studies (2, 3), the present series of experiments permits for the first time a comparison of the behavior in the precipitin reaction of antisera produced with the same antigen in the horse and in the rabbit. The data summarized in the tables indicate that the

TABLE II

Addition of S III to Serum 3.51

Amount S III added	Antibody N precipitated	Antibody N calculated from equation	Experimental ratio N precipitated to S III precipitated	Character of precipitate	Tests on supernatants
mg.	mg.	mg.			

Course 1. Serum diluted with 5 volumes saline. 1.0 ml. samples used

0.02	0.217	0.23	10.9		Excess A
0.04	0.424	0.41	10.6		Excess A
0.06	0.544	0.54	9.1		Excess A
0.08	0.617	0.62	7.7		No A or S
0.10	0.664*	0.66	6.6		No A or S
0.12	0.680		5.8†		Trace S
0.15	0.687				Excess S
0.177	0.682		4.1†		Excess S
0.20	0.696				Excess S
0.30	0.660				Excess S
0.50	0.619				Excess S
1.00	0.255				Excess S
1.50	0.064				Excess S

Equation: Mg. antibody N pptd. = $12.7 S - 61.1 S^2$. S max. = 0.104 mg.

A max. = 0.661 mg. N

Course 2. Serum diluted with equal volume saline. 1.0 ml. samples used

0.02	0.251	0.27	12.5	Op., Fl.	Excess A
0.04	0.436	0.44	10.9	Op., Fl.	Excess A
0.06	0.550	0.56	9.2	Op., C.	Trace A
0.08	0.588	0.60	7.4	Tl., C.	Trace S
0.10	0.614			Jelly	Excess S
0.12	0.644		5.6†	Jelly	Excess S
0.20	0.638			Jelly	Excess S
0.50	0.504*			Jelly	Excess S
1.00	0.124			Jelly	Excess S
1.50	0.004				

Equation: Mg. antibody N pptd. = $14.5 S - 87.1 S^2$. S max. = 0.084 mg.

A max. = 0.600 mg. N

Op. = opaque; Fl. = flocculent; C. = compact; Tl. = translucent.

* One determination only.

† Calculated after analyzing supernatant for S according to (9) and deducting amount found from that used.

TABLE II—*Concluded*

Amount S III added	Antibody N precipitated	Antibody N calculated from equation	Experimental ratio N precipitated to S III precipitated	Character of precipitate	Tests on supernatants
mg.	mg.	mg.			
Course 3. Serum diluted with 0.5 volume saline. 1.0 ml. samples used					
0.02	0.292	0.30	14.6	Op., Fl.	Excess A
0.04	0.554	0.54	13.9	Op., Fl.	Excess A
0.06	0.766	0.74	12.8	Op., Fl.	Excess A
0.08	0.866*	0.88	10.8	Op., Fl.	Excess A
0.10	0.926	0.97	9.3	Op., C.	Excess A
0.15	0.982*	0.98	6.5	Op., C.	No A or S
0.20	0.996				Excess S
0.50	0.940			Jelly	Excess S
1.00	0.602			Jelly	Excess S
1.50	0.362			Jelly	Excess S
2.00	0.336			Jelly	Excess S
Equation: Mg. antibody N pptd. = 16.1 S - 63.9 S ² . S max. = 0.126 mg. A max. = 1.015 mg. N					

Course 4. 1.5 ml. samples undiluted serum used

0.02	0.298	0.30	14.9		Excess A
0.04	0.518	0.51	13.0		Excess A
0.06	0.652*	0.66	10.9		Excess A
0.08	0.724	0.72	9.1		No A or S
0.50	0.704				Excess S
1.00	0.386				Excess S
1.50	0.070				Excess S
Equation: Mg. antibody N pptd. = 16.7 S - 96.5 S ² . S max. = 0.087 mg. A max. = 0.722 mg. N					

Calculated to 1.000 mg. of antibody N, these four equations become:

For 3.51₁: mg. antibody N pptd. = 12.7 S - 40.3 S²

" 3.51₂: mg. antibody N pptd. = 14.5 S - 52.6 S²

" 3.51₃: mg. antibody N pptd. = 16.1 S - 64.8 S²

" 3.51₄: mg. antibody N pptd. = 16.7 S - 69.7 S²

For serum 3.50, calculated to 1.000 mg. of antibody N, the following were found:

" 3.50₁: mg. antibody N pptd. = 14.9 S - 46.2 S²

" 3.50₂: mg. antibody N pptd. = 15.6 S - 60.8 S²

" 3.50₃: mg. antibody N pptd. = 16.8 S - 70.6 S²

reaction between homologous polysaccharide and antibody in rabbit Type III antipneumococcus sera follows the same general course as

in sera produced in the horse (1) and may also be quantitatively described by expressions of the form of Equation 1 derived from the mass law.

From Table I it will be noted that the effect of temperature on the S III-antibody reaction is much the same in the sera of the two animals in question (*cf.* (1) for the reaction with horse antibody), less antibody being precipitated at 37° than at 0°. However, the solubility of the specific precipitate at 0° is considerably greater in the rabbit antisera than in horse antisera and is about the same as that of the egg albumin-antibody precipitate (3). Subject to this correction, the amount of antibody precipitated does not depend on the final concentrations of the reactants, but on the relative proportions in which they are mixed, just as in the other systems studied.

In Table II are given data on the addition of increasing amounts of S III to sera obtained from the same rabbit after four successive courses of intravenous injections of formalinized Type III pneumococci. While inconstant results were obtained with earlier preparations of S III and the equivalence zone was characterized by the simultaneous presence of both polysaccharide and antibody, it will be noted that with S III prepared without the use of heat, strong acid, or alkali, as in (5), neither antibody nor polysaccharide could be detected in supernatants in the equivalence zone. When the ratios of antibody N to S III precipitated in the region of excess antibody were plotted against S III and the best line, calculated by the method of least squares, was drawn through the points, the equation of the line

$$\frac{N}{S} \text{ precipitated} = 2R - \frac{R^2}{A} S \dots\dots\dots [2]$$

described the behavior of the serum in the region of excess antibody and was converted into Equation 1 by multiplying through by S. Equations of this form, which give the amount of antibody N precipitated by any quantity of S up to the maximum, are given below the data for the serum from each course. Comparison of the found and calculated antibody values (columns 2 and 3, Table II) shows close agreement.

At the foot of Table II the equations for the four courses are compared at the same antibody content. It will be noted that R increases during immunization, as had previously been noted in the case of a rabbit injected with egg albumin (3). As also noted at the foot of Table II, R increased similarly during the immunization of another rabbit, 3.50. The data in Table III show that the amplitude of the equivalence zone does not appear to increase as definitely on progressive immunization as in the egg albumin system. It

TABLE III
Equivalence Zone Ratios of Antibody N to S in Precipitate

Serum	Observed R at A excess end of zone	R from equation	Observed R at S excess end of zone
Rabbit 3.50 ₁ Type III.....	7.9	7.5	6.3
" 3.50 ₂ " ".....	8.9	7.8	6.4
" 3.50 ₃ " ".....	10.5	8.4	6.8
" 3.51 ₁ " ".....	(8.5)	6.4	(6.1)
" 3.51 ₂ " ".....	9.2	7.3	7.4
" 3.51 ₃ " ".....	(8)	8.1	(6)
" 3.51 ₄ " ".....	(10)	8.4	
" 3.48 ₁ " ".....	(5.5?)	6.5	(5)
Mean.....		7.6	
Corresponding R from antipneumococcus III horse serum 792 at 0° with S III prepared according to Ref. 5.....	11.3	10.6	6.1
Rabbit 3.70 Type I.....	(3.0)	2.7	(2.7)
Corresponding R from horse antipneumococcus I solu- tion at 0° with S I prepared according to Ref. 5.....	(6.8)	7.2	(4.0)

Values in parentheses deduced from nearest actual determinations.

appears probable, nevertheless, that the observed increases in R in the later stages of immunization are due to the formation of antibody reactive with additional chemical groupings on the S III molecule which did not react with the antibody produced in the earlier stages of immunization.

From Table IV it will be seen that the relation

$$\text{mg. S III precipitated} = 2R' A - \frac{(R')^2}{S} A^2 \dots \dots \dots [3]$$

in which S is the total amount of S III added and R' is the ratio of S III to antibody N in the precipitate at the S III excess end of the equivalence zone, holds moderately well for the region of polysaccharide excess up to the beginning of the inhibition zone. As will also be noted from Table II, the extent of the region of maximum precipitation is quite limited, and the inhibition zone begins with smaller amounts of specific polysaccharide than necessary for Type III antipneumococcus horse sera. However, in the region of S III excess there appears to be little difference in the composition of the specific precipitate in the rabbit and horse sera, as the ratios of the components are much the same in both (Tables II and III, also Table III, reference 1), except possibly toward the beginning of the inhibition zone, at which N:S ratios as low as 3.8 and 3.5 were found for

TABLE IV

Calculated and Found Values of S III Precipitated in Region of Excess Polysaccharide

Serum	S III added	S III found in precipitate	S III calculated in precipitate	Serum	S III added	S III found in precipitate	S III calculated in precipitate
	mg.	mg.	mg.		mg.	mg.	mg.
3.48 ₁	0.167	0.163	0.167	3.50 ₃	0.118	0.117	0.118
($R' = 0.2$)	0.200	0.192	0.193	($R' = 0.147$)	0.177	0.171	0.156
($A = 0.81$)	0.225	0.211	0.207	($A = 0.79$)	0.236	0.199	0.175
	0.250	0.232	0.219				

rabbit sera 3.50₃ and 3.48₁, respectively. On the other hand, in the equivalence zone and in the region of excess antibody (Tables II and III, and Table I, reference 1) the combining ratios of the components are approximately half again as high in Type III horse sera as in rabbit sera, and the difference may become even greater when antibody is present in large excess. In these regions of the reaction range a given amount of horse Type III pneumococcus anticarbohydrate precipitates less S III than does the same amount of rabbit antibody. In the administration of serum therapeutically this advantage of Type III rabbit serum² over horse serum would be lost as far as the

² Horsfall, Goodner, and MacLeod (10) have reported a larger number of protective units, also, per milligram, of antibody N in rabbit Type I antipneumococcus serum than in horse serum.

initial doses are concerned, since these would be given in the region of excess S III, in which the combining ratios are much the same.

The differences in combining ratios in the rabbit and horse antisera are paralleled by differences in the character and appearance of the precipitate. With antibody in large excess there are formed opaque flocculcs (Table II) which are easily broken up and resuspended. With less antibody in excess the precipitate becomes more and more compact, and at the S III excess end of the equivalence zone an almost transparent jelly is formed. This becomes entirely transparent when there is a greater excess of S III. In the horse antisera the precipitate remains flocculent throughout the entire range of antibody excess, becomes compact in the region of slight S III excess, and gradually turns more jelly-like and transparent as the inhibition zone is reached. The appearance of the precipitates, however, appears to depend on the N:S III ratio rather than on any species difference, since precipitates from the horse or rabbit with the same N:S III ratios are scarcely distinguishable.

The effect of increased salt concentrations in reducing the quantity of antibody precipitated by a given amount of S III is greater in the rabbit S III-antibody system than with the horse antibody (Table I, reference 11). Consequently, specific precipitates from rabbit antisera were found to give larger yields of highly purified antibody when dissociated with strong salt solutions (12).

In the experiments reported it has been shown that the behavior of Type III antipneumococcus rabbit sera over a large part of the precipitin reaction range may be described by means of the equations given with a degree of accuracy comparable with that found for the corresponding horse sera. The constants for these equations can be determined by the micro estimation of the amount of nitrogen specifically precipitable by a small number of properly chosen, accurately measured quantities of S III (*cf.* (4)). From the limited data at hand, summarized in Tables V and III, it would appear that the same relations apply to Type I antipneumococcus rabbit sera³ in the only region investigated, that of excess antibody. Until the Type I pneumococcus specific polysaccharide used was prepared according to (5) it was found impossible to obtain accurate data as

³ Also horse sera (unpublished data).

to the composition of the specific precipitate, since S and A were found together in the supernatants over a large part of the reaction range both in horse and rabbit antisera.

A notable difference between the S I-antibody system (Table V) and that of S III in both the horse and the rabbit sera is found in the far lower nitrogen to polysaccharide ratios in the Type I specific precipitate than in the Type III precipitate. The difference in the ratios in the horse and rabbit sera appears to be even greater in the Type I system than in that of Type III.

TABLE V

Addition of S I to Type I Antipneumococcus Serum 3.70

Amount S I added	Antibody N precipitated	Antibody N calculated from equation	Experimental ratio N precipitated to S I precipitated	Tests on supernatants
mg.	mg.	mg.		
0.025	0.139*	0.13	5.6	Excess A
0.050	0.226	0.23	4.5	Excess A
0.075	0.308	0.31	4.1	Excess A
0.100	0.371	0.37	3.7	Excess A
0.125	0.394	0.41	3.2	Excess A
0.150	0.431	0.44	2.9	No A or S
0.200	0.465			Excess S
0.250	0.480			Excess S
0.300	0.483			Excess S

Equation: Mg. antibody N pptd. = $5.4 S - 16.7 S^2$. S max. = 0.162 mg.

A max. = 0.437 mg. N

Excess S I old (13) precipitated 0.08 mg. antibody N from this serum, S I acetyl (14) 0.204 mg. antibody N. With smaller amounts of S, the equivalence zone in both instances showed the presence of both A and S, instead of neither, as with S I prepared according to (5).

* S I nitrogen deducted from all values in this column.

While it is probable that the larger molecular weight of pneumococcus anticarbohydrate in the horse than in the rabbit (15, 16) is in part responsible for the higher nitrogen to polysaccharide ratios in specific precipitates from horse sera, the low ratios observed in both horse and rabbit Type I specific precipitates are not necessarily due to a higher molecular weight for the Type I specific polysaccharide (S I) than for S III. The relation between equivalence point ratio and molecular weight which Hooker and Boyd (17) sought to estab-

lish can scarcely apply to the specific polysaccharides. Indeed it is difficult to believe that the exceedingly viscous solutions yielded by the specific polysaccharides of pneumococcus as now prepared (5) are not the result of a relatively high molecular weight as well as due to the Coulomb forces (18) caused by the large number of acid groups in the molecules of substances such as S I and S III. The low molecular weights indicated some years ago (19) were found with the aid of material now known to have been partially degraded (5). Moreover, the assumption of a low polysaccharide molecular weight is not necessary for our quantitative theory of the precipitin reaction. According to current views as to polysaccharide structure, the pneumococcus specific polysaccharides, also, may be considered to have coil-shaped, zigzag, or rod- or thread-shaped molecules and to derive their immunological multivalence from recurrent chemical groupings characteristic for each pneumococcus type. With such a structure the factor determining combining ratios with antibody would not be the molecule as a whole, but the minimum chain length capable of reacting with a single molecule of antibody. This quantity may be calculated from the combining ratios in the region of extreme antibody excess, in which the polysaccharide may be considered to be "saturated" with antibody. Use is made in this calculation of the recent tentative assignment of molecular weights of about 150,000 and 500,000 to pneumococcus anticarbohydrate in the rabbit and horse, respectively (15).

For the three Type III antipneumococcus rabbit sera studied in detail, the mean value of $2R$, the maximum calculated antibody N:S III ratio, was 13.5. It is seen that this number is between one-half and one-third the maximum calculated ratio (about 32, Table IX, reference 1) for horse sera under corresponding conditions. If these figures be multiplied by 6.3, the values 85 and 200 are obtained for $2R$ expressed as the antibody protein:S III ratio. Dividing these numbers into 150,000 and 500,000, the tentative rough estimates of the molecular weights of anticarbohydrate globulin produced in the rabbit and in the horse (15), respectively, one obtains 1800 and 2500 as the weight of the minimum chain lengths of S III reactive with an antibody molecule in each antiserum. Hypothetical units of this size would correspond to five to eight aldobionic acid groupings (20).

A similar calculation with the data at hand from only two sera with $2R = 5.4$ and 14.4 for rabbit and horse Type I antipneumococcus sera, respectively, gives 4400 and 5500 for the minimum reactive chain lengths of S I, values about twice as great as those for S III. In accord with this, experiments with numerous Type I rabbit and horse antisera have shown that far more S I (5) is required to give maximum antibody precipitation than is necessary for the precipitation of antibody from Type III sera with S III. While any theoretical interpretation of these figures would be premature, it is at least evident from these values and the other ratios given that all through the reaction range, it requires roughly twice as much horse or rabbit antibody to combine with a given amount of S III as with the same weight of S I. Since in addition Type III pneumococcus produces far more S than does the Type I organism, both factors would probably contribute to the greater success of serum therapy in Type I pneumonia.

Equivalence zone ratios in the Type III rabbit antisera range from about 9 to about 6 at the two ends of the zone, corresponding roughly to one molecule of antibody for every 2600 to 3900 of polysaccharide weight units. In the inhibition zone, with much S III in excess, ratios of about 3 are indicated in the precipitate, corresponding to about 7900 units of S III per molecule of antibody. If the composition of the precipitate at the extreme antibody excess end of the range be represented by \bar{S}_3A , in which \bar{S} represents the minimum combining weight of S III, and A a molecule of antibody, the composition of the precipitate in the equivalence zone would range roughly from \bar{S}_3A_2 to \bar{S}_2A , while in the inhibition zone \bar{S}_4A would be reached. Since it has been shown (21) that the soluble compound formed in the inhibition zone contains one more unit of S III than the immediately preceding insoluble one, the composition of the soluble complex would approximate \bar{S}_5A .

Applying similar reasoning to horse anti-S III globulin, the equivalence zone ratios (Table VI, reference 1, and Table III) would correspond roughly to the limits of composition \bar{S}_3A and \bar{S}_6A , while the inhibition zone complex would be of the order of $\bar{S}_{10}A$.

While these figures are only extremely rough approximations which will necessarily have to be revised, they at least give the order of magnitude of the equivalent combining ratios of the two components

of the specific precipitate at definite reference points or zones in the reaction range. The order of magnitude of the combining proportions appears sufficiently small to justify the classical chemical treatment given, although the tentative formulas are not necessarily those of single chemical components. Figures now available for antigen-antibody systems which have been studied will be reported in a subsequent communication.

SUMMARY

1. The reaction between the specific polysaccharide of Type III pneumococcus and homologous antibody in rabbit sera is quantitatively accounted for by expressions similar to those derived from the mass law for the corresponding horse sera. Preliminary data are also given for the Type I reaction.

2. Differences and similarities of the reaction with antibodies produced by the two animals are discussed.

3. Calculations are made of the equivalent composition of the specific precipitate at various reference points in the reaction range.

4. Certain theoretical and practical implications of the findings are pointed out.

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CHEMICAL PROPERTIES OF THE PURIFIED SPREADING FACTOR FROM TESTICLE

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PLATE 23

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A factor having the property of enhancing infections and increasing tissue permeability has been shown to exist in testicle extracts (1, 2), and in lesser concentration, in extracts of mammalian organs (3). Similar factors have been found to occur in abundance in invasive bacteria (4), in certain poisonous animal products (5), in leech extracts (6), and in many malignant tissues (7). However, the striking similarity of action of these products on skin permeability does not necessarily imply that the active substances obtained from these different sources are related chemically, or that the same mechanism is involved in the production of the spread.

The study of the phenomenon would be made easier if the chemical nature of the factors of various derivation could be established. The action of the spreading agents on the tissues, and the particular response which results in the spread are not clearly understood. It has been shown that enhancement of infection and extensive spread of particles in the dermis could be induced by solutions of azoproteins (8), but no other chemical has been found which exhibits similar properties. There is evidence that the spreads produced under the influence of azoproteins, on the one hand, and of active tissue extracts, on the other hand, proceed from an entirely different mechanism (6).

The chemical properties of the spreading factor occurring in testicle extracts have been investigated. The results presented in this paper suggest that the spreading factor extracted from testicle is a protein.

Material and Method

Test for Spreading Activity.—The power of an extract to spread was determined by measuring the area of diffusion 24 hours after injection of 0.5 cc. of the extract, intradermally in the rabbit. Higgins' India ink, diluted 1:3 with water and filtered through a Berkefeld candle was used as indicator, and 0.25 cc. of it was added in the syringe to the test solution before injection. It is probable that the extent of the diffusion, as indicated by the spread of the ink particles, is not an accurate measure of the spreading power of the solution tested. The bigger the particle, the sooner it is likely to be retained in the tissue spaces; whereas the spreading agent itself or the substances dissolved with it continue to spread beyond the limit reached by the ink. The India ink was utilized as indicator because of the sharp outline it usually gives to the area of diffusion, and because no more than a relative measure of the potency of the various fractions of the testicle extract was judged necessary in the work. A control injection was always made in the rabbit treated, 0.5 cc. of 0.9 per cent NaCl being substituted for the test solution, and injected in a corresponding situation on the other side after the addition of India ink.

The area of spread appears to depend to a great extent on the individual permeability of the rabbit skin, which is apt to vary greatly from one animal to another. In order to take these variations into account, the area of spread produced by the extract was divided by the area of spread produced by the saline control. The resulting figure is more or less independent of the individual permeability of the skin.

Source of Spreading Factor and Preparation of Extracts.—A study of the distribution of the spreading factor in the animal body has shown that practically every one of ten organs extracted yields, in varying amount, an agent increasing skin permeability (3). Among mammalian organs, the testicle gave with regularity the most active preparations. Rabbit and rat testicles yielded extracts with a greater spreading power than did bull and guinea pig testicle extracts. Bull testicle was selected for the present work because abundant material could be secured at low cost. In some early tests rat testicles were used with the same general results.

Before extraction the bull testicle was trimmed of its membrane and passed through a meat grinder. The resulting pulp was found to contain 13.4 per cent of solid matter. It was ground with sand and an amount of water added equal in weight to the pulp. After a short period of contact, the undissolved fraction was separated by centrifuging the mixture at 3,000 R.P.M. for 15 minutes. After filtration through paper the dry weight of this extract averaged 42.8 mg. per cc.

EXPERIMENTAL

Solubility of the Spreading Factor in Acid Solution.—McClean has reported that acidification of the water extract of testicle tissue would

precipitate out a large portion of the inert protein material without much reduction of the spreading power of the solution, if the reaction is not reduced below pH 5.0. A further reduction to pH 4.0 shows no immediate effect, but incubation at 37°C. for 24 hours results in suppression of the spreading power of the solution. The factor was immediately destroyed at pH 3.0 or pH 2.0 (2). In studying the properties of the testicle extract, it was determined that the factor may become attached to the bulky precipitate and be removed from solution. But if the acidification is brought about cautiously with buffer, the resulting finely divided precipitate may be removed without loss in the concentration of the spreading factor. With this evidence that the factor is soluble in weak acids, an attempt was made to extract it directly by acid solution, thus avoiding much of the inert material soluble in water.

Four bull testicles, trimmed of the connective tissue, were minced, and the resulting 957 gm. of pulp ground with sand and treated with an equal volume, *i.e.* 957 cc., of 0.1 N acetic acid. After standing overnight in the ice box, the material was centrifuged and the supernatant fluid filtered through paper. The resulting fluid was clear, reddish, and had a reaction of pH 4.7. The dry weight of similar acid extracts was found to vary between 23.2 and 25.6 mg. per cc. This factor, free of the major part of the inert proteins, tested on rabbits proved to have a spreading power of the same order as that of the water extract.

A further experiment was carried out to determine the solubility in glacial acetic acid. The material tested was 1 gm. of dry powder of the purified factor. This was taken up in 20 cc. of glacial acetic acid, shaken for 20 minutes, and the undissolved part separated by centrifugation. The supernatant fluid was treated with 4 volumes of acetone, a procedure which, as will be shown later, precipitates the active spreading fraction. The precipitate was washed with more acetone and desiccated. The sediment insoluble in glacial acetic acid was also washed with acetone and dried. These two fractions, taken up in water and tested in the skin of rabbits, proved to possess about equal spreading properties.

Precipitation of the Spreading Factor by Organic Solvents.—McClean has reported that the factor can be precipitated out of an aqueous solution by 5 volumes of a mixture composed of 2 parts of alcohol and 3 of ether. Alcohol alone would bring down the active agent, but had the disadvantage of precipitating large amounts of inert material which interfered with further purification. There was no

indication of solubility of the active fraction in boiling chloroform or petroleum ether (2). These observations of McClean have been confirmed and extended. The spreading factor, either in the crude extract or in purified form, proved to be insoluble in ether, alcohol, acetone, chloroform, benzene, and pyridine. Acetone, under proper conditions, was found to be an excellent precipitating agent for the factor, removing it from aqueous solution quantitatively.

An acetic acid extract of bull testicle was treated with 4 volumes of acetone and the mixture left in the ice box overnight. The flocculent precipitate was collected on a Büchner funnel and washed with acetone until free of water. After drying in air, the yield of dry powder amounted to about 1.49 gm. per each 100 gm. of fresh tissue used for extraction. This fine white powder proved about 77 per cent soluble in water. This powder, kept in a dry state either at room temperature or in the ice box for 4 years, loses none of its spreading property, and there is evidence even that the spreading power increased with the aging of the powder.

Solutions prepared by extracting 3 gm. of powder with 100 cc. of distilled water gave detectable spread in the skin of a rabbit in dilutions as high as 1:100,000. Dilutions containing 0.0015 mg. solids per cc. still showed a noticeable effect on the permeability of the skin.

Fractionation by Ammonium Sulfate.—It was noted that the factor obtained by the above method was soluble in half saturated ammonium sulfate but was precipitated in saturated solution. This property was used in the further attempts to secure a purer product.

The dry acetone precipitate was reextracted with distilled water in the proportion of 4 gm. to 25 cc. of solvent. The insoluble part was discarded by centrifugation and filtration. Ammonium sulfate solution was added to the filtrate up to 0.4 saturation, and the mixture allowed to stand in the ice box overnight. The resultant precipitate was discarded and the solution brought to full saturation by the addition of crystals of ammonium sulfate, and again allowed to stand overnight. The resulting brownish precipitate was easily and completely soluble in water. The two precipitates obtained, that from 0.4 saturation and that from full saturation, were taken up in water and dialyzed, in preparation for the test in rabbit skin, along with the third fraction which remained in solution in the saturated ammonium sulfate. The first and third fractions were devoid of any spreading activity. On the other hand, 5 mg. of the fraction precipitated by full saturation gave an area of diffusion in the skin of 49 to 54 sq. cm. Dilutions as high as 1:10,000, corresponding to the injection of 0.0005 mg., were sufficient to produce increase in the permeability of the skin.

Preparation of the Spreading Factor in Purified Form.—On the basis of the foregoing observations, a method has been evolved for the purification of the spreading factor in a stable form.

Method.—Bull testicle is extracted, as described above, with an equal volume of 0.1 N acetic acid. After filtration through paper, the factor is precipitated out by 4 volumes of acetone and this precipitate dried. The precipitate is re-extracted with distilled water in the proportion of 25 cc. per gm. of the powder, and the insoluble part removed by centrifugation and filtration. The filtrate is mixed with an equal volume of saturated ammonium sulfate and the precipitate discarded. The clear filtrate is now brought to saturation with ammonium sulfate, and the resulting precipitate collected on folded filter paper. This fraction is very soluble in water, and can be taken up in a volume of water considerably less than the original solution and dialyzed until free of sulfate.

The purified fraction is a clear fluid, light brown in color. It is not precipitated by weak acid or by hydrochloric acid. Concentrated nitric acid produces a precipitate which does not redissolve on heating. It is precipitated by trichloroacetic acid. Acetone, alcohol, and ether precipitate the substance from solution.

The biuret, Millon's, xanthoproteic, ninhydrin, and diazo reactions are positive. The sodium nitroprusside reaction is negative, but the solution gives a positive test for sulfur when lead acetate is added after boiling with alkali. No precipitate is produced by copper sulfate. Mercury, gold, and platinum chloride, and silver nitrate cause precipitation of the solution.

The nitrogen content of the purified solution, determined by the method of Van Slyke (9), represented 14.2 per cent of the total solids.

The spreading power of the final product, tested in the rabbit skin, is shown in Table I. Injection of 0.05 mg. of the fraction gave a spread of 17.0 sq. cm. The limit of dilution at which some effect on skin permeability could be detected was 1:100,000, corresponding to the injection of 0.00005 mg. substance. Results of tests made with less purified fractions are also shown in Table I.

The purified fraction, injected together with a suspension of virus (vaccinia) or bacteria (staphylococcus), had the enhancing effect normally exhibited by the plain testicular extract. The purified extract was devoid of any hemolytic action on red blood cells, in contrast to the crude testicular extract (10).

Properties of the Spreading Factor

Some of the properties of the spreading factor were brought out in the methods used in its preparation. To arrive at some idea of its chemical nature, other properties were investigated.

Dialysis and Filtration.—As noted in the above method, the factor failed to pass through semipermeable membranes. Solutions of the acetone precipitate were submitted to dialysis in collodion or cellophane sacs for 4 to 8 hours, using the technique of Kunitz and Simms (11). The loss in diffusible matter amounted to as much as 71.8 per

TABLE I

Spreading Power and Solid Content of Active Solutions Obtained by Successive Fractionation of Bull Testicle Extract (Recorded 24 Hours after Injection)

Solutions tested	Total solids in solution	Amount injected for the test	Area of spread of 0.5 cc. test solution plus 0.25 cc. India ink suspension	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink suspension (control)	Ratio of active spread to spread of control
	mg. per cc.	mg.	sq. cm.	sq. cm.	
Water extract	43.2	21.6	34.6	5.2	6.6
0.1 N acetic acid extract	25.4	12.7	38.5	5.0	7.7
Water extract of acetone precipitate	23.7	11.8	40.3	4.8	8.4
Fraction precipitated by saturation with ammonium sulfate, dialyzed solution	17.7	8.8	56.6	5.9	9.6
“ “	10.0	5.0	41.0	5.9	6.9
“ “ diluted 1:10	1.0	0.5	29.7	5.9	5.0
“ “ diluted 1:100	0.1	0.05	17.0	5.9	2.9

cent of the total dry weight, but no reduction in the spreading power was detected. The dialyzed solution was completely filterable through Berkefeld candles, with no loss in spreading power or in solids.

Effect of Heat.—Extracts of fresh testicle tissue or of acetone precipitates are inactivated by heating at 100°C. for 5 minutes. There is evidence that this is due, at least partly, to adsorption of the factor on the abundant precipitate produced by heating. It was found that acidification of the purified material delayed, but did not completely prevent, inactivation by heat. Thus a solution of the acetone

precipitate at pH 4.5 was little affected by immersion for 5 minutes in boiling water, but 10 minutes or more at 95°C. resulted in almost complete inactivation. On aging, the powdered acetone precipitate was found to undergo certain changes which tended to increase the stability of the factor for heat. However, none of the preparations

TABLE II

Effect of Proteolytic Enzymes on the Spreading Activity of Testicle Extracts

Testicle fraction tested	Enzyme preparation used	pH of enzyme mixture and of control during incubation at 37°C.	Area of spread of 0.5 cc. test solution plus 0.25 cc. India ink indicator (24 hrs after injection)						
			Saline (control)	Enzyme added before injection	Extract incubated without enzyme	Extract incubated with enzyme at pH 6.2	Extract in ice box at pH 2.0 during experiment	Extract incubated with enzyme at optimum pH	
								2 hrs. at 37°C.	4 hrs at 37°C.
Acetone precipitate, extract dialyzed	Crystalline trypsin in 95 per cent glycerin (1932)	8.0	sq. cm. 3.0	sq. cm.	sq. cm. 33.0	sq. cm.	sq. cm.	sq. cm. 3.2	sq. cm. 3.0
Fresh bull testis, Berkefeld filtrate	" "	7.6	3.7	40.2	38.8			23.9	10.2
Ammonium sulfate precipitate, Berkefeld filtrate	Crystalline trypsin dried with MgSO ₄ (1936)	7.7	6.2		44.1	21.8		10.0	7.3
" "	Crystalline carboxypolypeptidase	8.0	7.5		38.5				37.6
" "	Crystalline pepsin in 95 per cent glycerin (1933)	2.0	7.5		33.0	37.4	30.0		7.8

was found to withstand a temperature higher than 60°C. for a prolonged period. These findings are at variance with those of McClean, who inferred from his results that the factor is probably heat-stable.

Resistance to Strong Acid.—In the purified form the factor is soluble in hydrochloric acid and withstands a hydrogen ion concentration

as high as pH 2.0. This resistance to strong acid made possible a study of the proteolytic action of pepsin on the testicular factor.

Effect of Proteolytic Enzymes.—Crystalline pepsin, crystalline trypsin,¹ and crystalline carboxypolypeptidase² were used (12–14). The results are summarized in Table II. The purified fraction, which will tolerate strong acids, was completely inactivated on incubation with pepsin at pH 2.0 for 4 hours, but the solution, kept at pH 2.0 without the enzyme or at pH 6.2 with the enzyme, was unaffected. The amount of pepsin added was about 1/50 the weight of the substrate. Trypsin, in the proportion of 0.0025 mg. protein nitrogen

TABLE III

Effect on Rabbit Skin of Concentrated Spreading Solutions (Recorded 24 Hours after Injection)

Solutions tested	Original volume reduced to	Area of spread of 0.5 cc. test solution plus 0.25 cc. India ink indicator	Area of spread of 0.5 cc. Ringer's solution plus 0.25 cc. India ink indicator (control)	Ratio of active spread to spread of control
		sq. cm.	sq. cm.	
Water extract of acetone precipitate.....	—	27.2	5.9	4.6
“ “ “ “ “	1/10	56.4	5.9	9.5
“ “ “ “ “	1/15	62.2	6.6	9.4
“ “ “ “ “	1/25	68.2	5.2	13.1
“ “ “ “ “	1/68	127.4	2.3	55.4
Ultrafiltrate.....	1/10 to 1/30	6.6	6.9	0.0

per mg. of dialyzed substrate at pH 8.0, completely destroyed or greatly reduced the activity of the solution when incubated at 37°C. for 2 to 4 hours. Trypsin inactivated also ordinary testicular extracts. This confirms a previous observation by McClean (2). The carboxypolypeptidase at pH 8.0 was without effect, and the solutions incubated with this enzyme for 4 hours showed no reduction of their spreading activity.

¹ These recrystallized enzymes were kindly supplied by Dr. Northrop. They were kept either dissolved in 95 per cent glycerin or dried in the presence of magnesium sulfate.

² The carboxypolypeptidase was kindly prepared by Dr. Anson. It was recrystallized twice and contained 1:1,000, by weight, of active trypsin.

Effect of Concentrated Preparations on the Rabbit Skin.—The effect of highly active preparations was tested. Solutions of the acetone precipitate were concentrated in Norton alundum thimbles lined with an 8 per cent solution of cotton in glacial acetic acid and placed under negative pressure (15). Preparations corresponding to a volume reduction of 10-, 15-, 25-, and 58-fold, respectively, were obtained and the spreading properties tested. Results are shown in Table III. None of the factor could be detected in the concentrated ultrafiltrate. The extensive spreads caused by the concentrated factor are illustrated in Figs. 1, 2, and 3. The more concentrated solution not only spread extensively, but the whole area involved appeared distinctly edematous. 24 hours after inoculation, fluid from the injected area was found to have collected by gravity in the subcutaneous tissue of the lower part of the abdomen. Some of the transudate was withdrawn by puncture. The liquid was transparent, almost colorless, and coagulated spontaneously. The fluid failed to increase the permeability of the rabbit skin. At no time during the formation and reabsorption of the edema was there any gross sign of inflammatory or irritative changes.

SUMMARY AND CONCLUSIONS

1. The factor responsible for the spreading property of testicle extracts was found to be soluble in water, in salt solution, and in acid media. It is relatively stable at high hydrogen ion concentrations, and it is not precipitated or inactivated by hydrochloric acid up to pH 2.0. The spreading substance is not soluble in acetone, ether, alcohol, chloroform, or pyridine. It is inactivated by crystalline trypsin and pepsin at the optimum pH of action of these enzymes. It is not attacked by a crystallized carboxypolypeptidase. The substance does not pass semipermeable membranes which retain proteins. The color tests for proteins are positive. At least 14.2 per cent of the fraction isolated is nitrogen.³ Taken together these properties are strong evidence that the testicular factor is a protein.

2. A method for the preparation of the spreading factor in relatively pure form is presented and discussed.

3. In addition to the spread, concentrated solutions of the testicular

³ These findings are not in agreement with the results recently published by Favilli (16).

factor are shown to produce a condition of the skin having the characters of edema.

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EXPLANATION OF PLATE 23

FIG. 1. Rabbit 4-99 (right side). Spread produced by intradermal injection of 0.25 cc. India ink suspension, plus: (back) 0.5 cc. water extract of acetone precipitate and (front) 0.5 cc. of the water extract concentrated 15-fold by ultrafiltration. The areas of spread 20 hours after injection were 27.2 and 62.2 sq. cm., respectively.

FIG. 2. Rabbit 4-82 (left side). Spread produced by intradermal injection of 0.25 cc. India ink solution, plus: A, 0.5 cc. Ringer's solution (control) and B, 0.5 cc. of an extract of acetone precipitate previously concentrated 25 times by volume. The areas of spread were 5.2 and 68.2 sq. cm., respectively.

FIG. 3. Rabbit 5-43. Spread produced by the intracutaneous injection of 0.25 cc. India ink solution, plus 0.5 cc. of acetone precipitate extract, concentrated 68 times by volume. The area of diffusion, measured 48 hours after injection, was 127.4 sq. cm. In this particular case the photograph was retouched with ink in order to provide enough contrast for photography. During the active spread most of the ink particles had been carried away towards the lower part of the abdomen. The edematous fluid infiltrating the connective tissue is responsible for the bulging of the skin.



STUDIES ON EXPERIMENTAL HYPERTENSION

III. THE PRODUCTION OF PERSISTENT HYPERTENSION IN MONKEYS (MACAQUE) BY RENAL ISCHEMIA*

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In a previous communication (1) a method was described for the production of persistent elevation of blood pressure in dogs by constriction of the main renal artery with a special silver clamp devised for the purpose. It was found that elevation of systolic blood pressure followed constriction of only one renal artery and that, after a varying period, this tended to return to a lower or even to the original level. In more recent experiments elevated systolic blood pressure has lasted as long as 7 months in a dog with only one renal artery constricted. This dog is still living, so it is not known whether or not the other kidney is normal. Following adequate constriction of both renal arteries, the blood pressure has remained elevated in most of the dogs, and some have now had hypertension for more than 4 years. The development of hypertension in dogs with experimental renal ischemia has now been amply confirmed by others (2-14). In most of the studies either systolic blood pressure alone or mean blood pressure was determined, but recently (5, 6) it has been shown that in dogs with renal ischemia, diastolic pressure also becomes elevated. This we have also confirmed, using the Kolls-Cash method (15) for the determination of diastolic pressure.

Since there are many points of similarity between the hypertension induced in dogs by renal ischemia and that which is associated with renal arteriolar disease in man, it is hoped that the investigations which are being carried out by the use of this method will throw some light on the pathogenesis of this type of hypertension in man. On that account it was considered of some importance to determine

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whether renal ischemia would induce the development of hypertension in an animal more closely related to man. There is no record of any attempt to produce hypertension in monkeys by any means. This publication deals with the développement of persistent hypertension in macaques following the production of experimental renal ischemia.

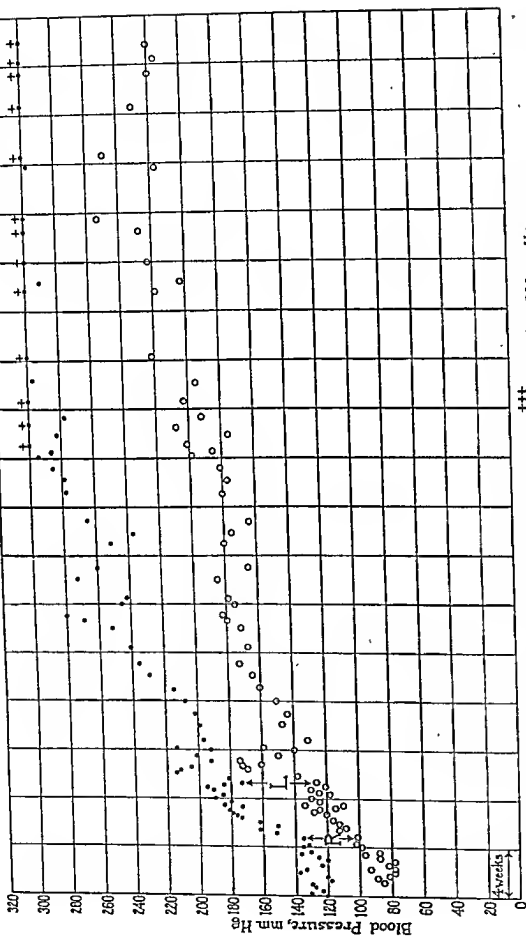
EXPERIMENTS

In this study the giant type of macaque was used. The animals were mature, but young. Their exact age was not known. They received a mixed complete diet and were kept in individual cages. Blood pressure before and after the production of renal ischemia was determined by two methods. The one used regularly was the Riva-Rocci method, with the cuff placed around the abdomen, and auscultation practised over the femoral artery in the groin, in the manner used by McGregor (16) for the rabbit. The readings therefore represent lower abdominal intraaortic systolic and diastolic pressures. The other method, which was used at irregular intervals, was the determination of so called mean pressure in the femoral artery by the insertion of a needle connected to a mercury manometer by means of a rubber tube filled with 2 per cent sodium citrate.

After a long control period, five giant macaques were anesthetized with ether and subjected to the operation previously described (1) for the production of bilateral renal ischemia in dogs. A smaller silver clamp was used. Only one renal artery at a time was constricted and the interval between the clampings varied from 6 weeks to 3 months.

RESULTS

Since the renal artery in the monkey is very small, the difference between partial constriction and actual occlusion of the artery is not very great. In one monkey (No. 4) the degree of constriction of both vessels was evidently too great. Systolic and diastolic pressures rose moderately after the clamping of the right renal artery, but it had returned to the original level at the time of the second operation 3 months later. After the clamping of the right renal artery this animal developed renal insufficiency and died in uremia 3 days after the operation. In another animal (No. 3) the degree of constriction was evidently insufficient for a permanent elevation of blood pressure, for it rose only slightly and soon returned to normal. For technical reasons, no attempt was made to increase the constriction of the renal arteries in this animal, a procedure which is frequently success-



TEXT-FIG. 1. Monkey 5. Female macaque, age about 1 year at beginning of experiment. Weight 4 kilos at beginning and 3 kilos at end of experiment. R, main artery, right kidney, severely clamped but not occluded. L, main artery, left kidney, severely clamped but not occluded.

Both systolic and diastolic pressures became elevated and remained elevated for 16 months after the clamping of the right renal artery. During the period of acute illness which lasted 9 days and which proved fatal, no determinations of blood pressure were made.

ful in dogs. Instead of this the left kidney was removed. The blood pressure again became moderately elevated, but the animal developed diarrhea and died 11 days after this operation. In the remaining three monkeys the systolic and diastolic pressures rose and remained elevated following the constriction of both renal arteries. In one monkey (No. 5), the elevation was very great. Text-fig. 1 is a record of the systolic and diastolic pressures of monkey 5 which showed the greatest elevation of blood pressure. By the cuff method, the lower abdominal intraaortic systolic blood pressure of this animal during the last 7 months of its life was regularly about 300 mm. Hg or more, and the diastolic blood pressure during this period was always above 200 mm. Hg. By the direct manometric method, the mean pressure in the femoral artery, which averaged about 120 mm. Hg during the preoperative control period, usually was between 250 mm. and 270 mm. Hg during the last 7 months of its life. This animal died of acute enteritis 16 months after the clamping of the first renal artery. During the control period in monkey 1 the average systolic pressure was 145 mm. Hg and the diastolic pressure was 92 mm. Hg. At the present time, 14 months after the clamping of the first renal artery and 11 months after clamping the second, the average systolic pressure is 196 mm. Hg and the diastolic 130 mm. Hg. Monkey 2 had an average systolic pressure of 128 mm. Hg and a diastolic pressure of 86 mm. Hg during the control period. At the present time, 17 months after the clamping of the first renal artery, and 14 months after clamping the second, the average systolic and diastolic pressures are 206 mm. Hg and 140 mm. Hg, respectively.

The report of the examination of the tissues of the monkeys will be included in a forthcoming report on the pathological changes in the tissues, especially in the blood vessels, of dogs that have had experimental hypertension for periods varying from a few weeks to 4 or more years. This report will be made when the number of animals studied has become sufficient to permit justifiable conclusions about the relationship between these changes and the hypertension.

SUMMARY

Persistent elevation of systolic and diastolic blood pressure due to renal ischemia has been produced in monkeys (macaque) by the same method previously used for this purpose in dogs.

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EFFECT OF INTENSE SONIC VIBRATIONS ON ELEMENTARY BODIES OF VACCINIA

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Interest in the effects of sonic energy on certain materials, including infectious agents, has increased during recent years. General reviews regarding the reaction of a number of substances to this type of energy have already been presented by Harvey (1) and by Chambers and Gaines (2). The fact that bacteria are broken up by supersonic vibrations induced us to ascertain whether elementary bodies of vaccinia are disrupted when subjected to sonic energy. We were particularly interested in the matter because disruption of the bodies would facilitate immunological studies in the virus field. The purpose of the present paper is to record the reaction of several kinds of vaccine virus preparations to intense sonic vibrations with a frequency of about 8900 cycles per second.

Methods and Materials

Sonic Energy.—A modified Peirce magnetostriction oscillator with a frequency of about 8900 cycles per second previously described and used by Chambers and Florsdorf (3) supplied the energy. The apparatus has an adequate cooling system and at no time during the experiments did the temperature of the virus preparations rise above 20°C.

Preparations of Vaccine Virus.—Elementary bodies of vaccinia were prepared according to the technic of Craigie (4) from the skin of rabbits infected with the C.L. strain of virus. Bodies obtained in this way are designated throughout the paper as prepared according to routine, whereas others, referred to as thoroughly washed, were subjected to further purification by means of additional washings. The latter preparations, although not absolutely pure, contained much less adventitious material than did those prepared in the routine manner.

Testicular vaccine virus was obtained from rabbits inoculated with the New

York City Board of Health strain. Both testes were inoculated and then removed from the animal 3 days later. After removal they were thoroughly ground; the finely divided tissue was suspended in 70 cc. of Locke's solution and centrifuged for half an hour at 2500 R.P.M. The supernatant fluid with its vaccine virus constituted the testicular virus used in our experiments.

0.25 cc. amounts of serial tenfold dilutions of the suspensions of elementary bodies and of the testicular virus emulsions were injected intradermally into rabbits in order to determine the degree of infectivity of the preparations. Each dilution was tested in two or more rabbits.

EXPERIMENTAL

Effect of Sonic Vibrations on Thoroughly Washed Elementary Bodies

Information regarding the effect that intense sonic vibrations have on thoroughly washed elementary bodies of vaccinia was obtained in the manner described in the following experiment.

Experiment 1.—Dermal pulp was obtained from 4 rabbits with 3 day vaccinal lesions. After preliminary dilution, which amounted to 30 cc. for the pulp obtained from each animal, the material was centrifuged for 2 periods of 5 minutes each in a horizontal centrifuge to remove coarse particles. The supernatant material placed in flat tubes having an inside diameter of 4 mm. and a capacity of about 4.5 cc. per tube was then spun in an angle centrifuge for an hour at 3500 R.P.M. The supernatant fluid from each tube was discarded and the sediment was resuspended in 4 cc. of a dilute citric acid-phosphate buffer solution, pH 7.2, and recentrifuged. Three such washings were carried out the first day. After resuspension following the last centrifugation in the angle machine the material was centrifuged in a horizontal machine for an hour at 2500 R.P.M. The resulting supernatant material was stored at $+5^{\circ}\text{C}$. Once during each of the next 3 days, the elementary bodies were sedimented in the angle centrifuge, resuspended in dilute buffer solution, and then spun in a horizontal centrifuge for an hour. Finally, the elementary bodies were sedimented in the angle centrifuge and resuspended in 44 cc. of the buffer solution. Preparations of this material stained according to Morosow's technic (5) showed many discrete elementary bodies of uniform size between which little or no granular precipitate was obvious. Three portions of the material consisting of 12 cc. each were respectively exposed to sonic vibrations for 15, 45, and 90 minutes; the remaining 8 cc. were used as a control.

The following results, depicted in Chart 1, were obtained in the above experiment. The infectious titer of the untreated suspension was 10^{-8} ; the material vibrated 15 minutes titered 10^{-4} ; that treated for 45 minutes titered 10^{-2} ; while that subjected to vibration for 90

minutes was not infectious in a 10^{-2} dilution, lower dilutions were not tested. The suspensions subjected to sonic vibrations were definitely more opalescent than was the control. There was a slight tendency for the elementary bodies to clump in the vibrated materials, but this clumping could not have accounted for the drop in titers because the opalescence and clumping were no more marked in the material treated for 90 minutes than they were in those subjected to vibrations for 15 and 45 minutes, respectively. Densitometer (6) readings,

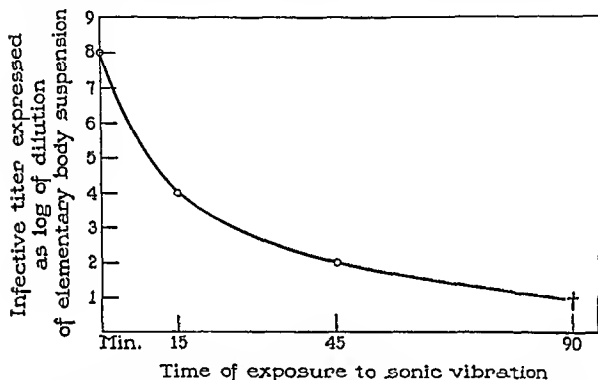


CHART 1. Graphic representation of inactivation of thoroughly washed elementary bodies by sonic vibration.

† The suspension of elementary bodies vibrated 90 minutes was inactive in a dilution of 10^{-2} ; lower dilutions were not tested.

stained preparations, and dark-field examinations gave no indications that any of the elementary bodies had become disintegrated as a result of subjection to sonic vibration; nevertheless, inactivation of the virus occurred.

Effect of Sonic Vibrations on Elementary Bodies Prepared According to Routine and on Testicular Vaccine Virus

Having found that thoroughly washed elementary bodies were inactivated without being broken up by sonic vibrations we decided

to test this kind of energy on bodies prepared according to routine and on testicular vaccine virus.

Experiment 2.—Dermal vaccine pulp was secured from 4 rabbits, and, after the preliminary dilution with 120 cc. of buffer solution, the coarse particles were removed from the suspension by means of centrifugation in a horizontal centrifuge. The elementary bodies were then washed 3 times in an angle centrifuge after which large particles or large clumps of elementary bodies were removed by centrifugation in a horizontal centrifuge for an hour. Four lots of 18 cc. each were treated in the following manner: one was vibrated for 2 minutes, another for 15 minutes, still another for 45 minutes, while the control was placed in the vibrating machine for 90 minutes without the oscillating current being active. The infectious titers of the 4 portions were the same, *viz.*, 10^{-7} .

A suspension of testicular vaccine virus as described above was divided into 4 portions, each consisting of 15 cc. The control portion was kept in the vibrating machine for 90 minutes without the current being turned on, while the other portions were vibrated for 15, 45, and 90 minutes, respectively. The titers, 10^{-5} , of the different portions were the same.

The results of the above experiment indicate that the elementary bodies prepared according to routine and vaccine virus in testicular emulsions were not inactivated by the sonic vibrations used. The vibrated materials became somewhat more opalescent than the controls; there was a slight tendency for the elementary bodies to clump but the clumping was not sufficient to influence the titers; an alteration in the hemoglobin resulted in a change in the color of the testicular emulsion.

Protective Action of Protein on Elementary Bodies

The decided differences in the action of sonic vibration on the 3 preparations of vaccine virus used in the preceding experiments led us to consider the possibility that such amounts of protein, as are present in suspensions of elementary bodies prepared according to routine or in testicular virus emulsions, might prevent the inactivation of vaccine virus by the type of energy used. The idea was tested in the following manner.

Experiment 3.—Elementary bodies were prepared in the routine manner from dermal pulp of 5 rabbits. The bodies were further washed in the angle centrifuge on 3 successive days but were centrifuged in the horizontal machine only after the third of the rewashings. The final preparation was diluted to 120 cc. Two

portions of 55 cc. each were removed; to one, 5 cc. of dilute buffer solution were added; to the other, 5 cc. of normal rabbit serum. Each of the 2 suspensions was divided into 4 equal parts; one part from each suspension was kept as a control, while one part from each suspension was vibrated 15, 45, and 90 minutes, respectively.

The results of titrations of the controls and the vibrated elementary bodies are shown in Table I and indicate that the suspensions of thoroughly washed elementary bodies without added serum were inactivated approximately to the same extent as were those in Experi-

TABLE I

Effect of Sonic Vibrations on Thoroughly Washed Elementary Bodies of Vaccinia; One Portion Was Suspended in Buffer Solution, the Other Was Suspended in Buffer Solution Containing Normal Rabbit Serum

Type of suspension	Time of vibration	Infective titer of elementary body suspension					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Elementary bodies suspended in buffer solution	No vibration	++++	+++	+++	++	++	±
	Vibrated 15 min.	++++	++	+	+	+	-
	Vibrated 45 min.	++++	++	+	+	-	-
	Vibrated 90 min.	+	-	-	-	-	-
Elementary bodies suspended in buffer solution plus normal rabbit serum	No vibration	++++	++++	++++	++++	++	±
	Vibrated 15 min.	++++	++++	++++	++++	+	±
	Vibrated 45 min.	++++	++++	++++	++++	++	-
	Vibrated 90 min.	++++	++++	++++	++++	+	-

± = papule approximately 3 x 3 x 1 mm.; + = papule approximately 6 x 6 x 1 mm.; ++ = papule approximately 10 x 10 x 1.5 mm.; +++ = 15 x 15 x 2 mm. with neighboring edema; ++++ = papule approximately 20 x 20 x 2 mm. with central hemorrhage and surrounding edema.

ment 1, whereas the titers of the suspensions containing serum were not significantly altered. Furthermore, no evidence was obtained that the elementary bodies in the inactivated material had been broken up.

Effect of Hydrogen Peroxide on Elementary Bodies in Preparations Containing Different Amounts of Protein

Results of work of Florsdorf, Chambers, and Malisoff (7) with an apparatus of the type used in our experiments indicate that the sub-

jection of pure water to sonic vibrations at atmospheric pressure for 45 minutes results in the production of approximately 400 micro-equivalents of hydrogen peroxide per liter. Inasmuch as the inactivation of the elementary bodies in our experiments was not due to their disruption by sonic vibrations it seemed of interest to determine whether small amounts of hydrogen peroxide would accomplish it.

Experiment 4.—The control materials used in the preceding experiment were employed. 0.5 cc. amounts of the suspension of thoroughly washed elementary bodies without added rabbit serum and of that to which rabbit serum had been added were respectively treated with equal volumes of (a) distilled water, (b) 1:150 dilution of U.S.P. 3 per cent hydrogen peroxide, and (c) 1:15 dilution of the 3 per cent hydrogen peroxide. Thus, the final concentrations of hydrogen peroxide in tubes b and c were 0.01 and 0.1 per cent respectively. The mixtures were allowed to stand for 90 minutes at room temperature, after which they were titrated for infectivity.

As in Experiment 3, the 10^{-6} dilution of the controls yielded a definitely positive reaction, while the 10^{-7} dilution gave only a questionable one. Elementary bodies in neither suspension were inactivated in significant amounts by exposure to a 0.01 per cent concentration of hydrogen peroxide. The 0.1 per cent concentration, however, reduced the titer of the suspension of bodies containing no rabbit serum to 10^{-2} , while the titer of the suspension with added rabbit serum was slightly infectious at a dilution of 10^{-5} , negative at 10^{-6} , and questionable at 10^{-7} .

Experiment 5.—An elementary body suspension was washed in the routine manner. Then a part of the suspension was further washed as in Experiment 3. Samples of both suspensions were treated with hydrogen peroxide as described in Experiment 4. It was found that the thoroughly washed elementary bodies were inactivated by 0.1 per cent hydrogen peroxide, while the bodies washed less thoroughly or according to routine were much more resistant to such treatment.

From the results of Experiments 4 and 5 it appears that 0.1 per cent hydrogen peroxide will inactivate a certain number of elementary bodies of vaccinia within a period of 90 minutes if they are not protected by adventitious protein.

DISCUSSION

The results of our experiments clearly indicate that adequately washed elementary bodies, obtained from rabbit dermal pulp, are inactivated by sonic vibrations with a frequency of about 8900 cycles per second. Small amounts of normal rabbit serum added to such preparations prevent inactivation. Furthermore, inadequately

washed elementary bodies and suspensions of testicular vaccine virus are not inactivated by the amount of energy employed.

Our findings agree with those previously reported regarding the action of supersonic vibrations on vaccine virus. Hopwood (8) found that calf lymph vaccine virus retained its activity in the presence of vibrations. In fact, it appeared to have had an increased infectious titer after treatment. This he attributed to a breaking up of particles of tissue which resulted in a greater dispersion of virus. Yaoi and Nakahara (9) noted that crude calf lymph virus was not inactivated by supersonic energy, whereas virus purified by adsorption and elution was inactivated. The Japanese workers suggested that impurities in the crude virus preparations prevented the inactivation of the agent by the vibrations. The results of our work clearly indicate that adventitious substances, particularly protein, in the preparations of vaccine virus protect the agent from inactivation by sonic vibrations. Of interest in this connection is the observation of Beckwith and Weaver (10) that protein is responsible for a diminished lethal effect of supersonic energy on bacteria in milk.

There appears to be no uniformity in the reaction of different viruses to sonic energy. Stanley (11) found that partially purified tobacco mosaic virus was less affected than was the virus in crude preparations of infectious plant juice. According to Scherp and Chambers (12), partially purified poliomyelitis virus and influenza virus in emulsions of infected mouse lung tissue were unaffected by intense sonic vibrations.

The disruption of bacteria (13) by high frequency sound waves led us to hope that the elementary bodies of vaccinia might also be broken up in such a manner. If it were possible to obtain their disintegration in this way, the solution of many immunological problems in this field would be facilitated. However, in spite of the fact that thoroughly washed elementary bodies were inactivated by vibration, no evidence was obtained that an appreciable number of them were broken up.

The mechanism by which sonic vibrations induce death and disruption of animal cells and bacteria is not fully understood (2). Moreover, it is not unlikely that the inactivation of vaccine virus is accomplished in a manner different from that operative in the de-

struction of cells and bacteria. At least no obvious disintegration of the elementary bodies was observed. Flosdorf, Chambers, and Malisoff (7) have found that approximately 400 microequivalents of hydrogen peroxide per liter were formed in distilled water subjected to sonic energy for 45 minutes in an apparatus of the type used in our experiments. This amount of hydrogen peroxide is less than 1 per cent of that necessary to inactivate the elementary bodies used in Experiments 4 and 5. Thus, while it seems justifiable to assume that hydrogen peroxide was not responsible for the loss of activity of the vibrated virus, one cannot avoid the implication that an oxidizing reaction played an important rôle in this inactivation, because it has been observed (7) that the oxidizing capacity of water during vibration, as determined by the oxidation of sodium bisulfite placed in the treated water, was in certain instances as great as 20 milliequivalents per liter per hour. This oxidizing capacity is of the same order as that of the hydrogen peroxide solutions (60 milliequivalents per liter) effective in inactivating the elementary bodies. In spite of this apparent correlation, no conclusions are warranted. However, it can be pointed out that the oxidizing ability of aqueous preparations during sonic vibration must be taken into consideration in explanations of the inactivation of elementary bodies of vaccinia by sonic energy.

SUMMARY

Thoroughly washed elementary bodies of vaccinia were inactivated by sonic vibrations with a frequency of about 8900 cycles per second; the inactivation was not accompanied by a disruption of the bodies. Adventitious substances, notably protein, prevented or hindered the inactivation. There is some evidence that oxidation might have played a rôle in the inactivation.

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VIRUSES OF POLIOMYELITIS

AN IMMUNOLOGICAL COMPARISON OF SIX STRAINS*

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During the past 5 years we have collected 6 strains of the virus of poliomyelitis and we have observed certain characteristics which differentiate some from the others.

Two of these strains (namely those isolated in New York and Connecticut in 1931) have already been compared with another pair of them representing more widely known strains of the virus (1). It has seemed justifiable, however, to pursue the comparisons further because the strain differences are considerable and poorly understood.

In poliomyelitis there is as yet no standard or recognized method whereby strains of the virus may be classified. Their properties are manifold and there is no indication as to which is of the greatest taxonomic importance. Thus, an outstanding property of a given strain is that of maximum virulence for the monkey on intracerebral inoculation. However, virulence by this route does not necessarily parallel virulence by the nasal or the cutaneous routes, to which latter fact one of our strains bears witness (2). But for purposes of classification, besides comparing virulence by different routes of inoculation, it is obvious that one should use the immunological approach, for it is generally the method of choice in classifying substrains of infectious agents. It has, nevertheless, been evident from the start that certain features of the immunology of experimental poliomyelitis render this approach difficult. It is recognized, for instance, that although

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sera of convalescent monkeys usually effect neutralization, irregularities have been reported (3-5). It is also recognized that the vaccination of monkeys with the virus results not only in the irregular production of immunity to infection, but in the irregular production of humoral antibodies, and, furthermore, that these two responses do not necessarily go hand in hand as the reports, among others, of Olitsky and Cox (6), Sabin and Olitsky (5), Hudson, Lennette and Gordon (7) and Kramer *et al.* (8) bear witness. In spite of these obvious objections one gathers, from Ayccock and Kagan (9) and Stewart and Rhoads (10), that the artificial immunization of monkeys appears to afford the best method available for the immunological comparison of strains, and, moreover, it has been successfully used by Kramer (11).

Consequently, following this method we vaccinated monkeys with the virus and later collected the presumably immune serum. This was saved until sera representing each strain were in hand. Then after a lapse of 12 to 18 months the appropriate neutralization tests were done. In the interim the vaccinated animals were tested by a series of intracerebral inoculations for resistance to homologous and heterologous strains. This last took 12 months to complete and the outcome was difficult to interpret, but the results of the neutralization tests seem reasonably clear and as such they form the subject of this report.¹

Methods

"Immunization" or "Vaccination" of Monkeys.—6 groups of from 2 to 4 monkeys each were inoculated intracutaneously with each of our 6 strains of virus. At least 1 animal was inoculated with live virus and, following the suggestion of Brodie (12), 1 animal with formalinized virus. This was prepared in saline from a 20 per cent suspension of spinal cord ground with sand in a mortar. The suspension was centrifugalized at moderate speed, 1600 R.P.M., for 6 to 8 minutes, and to 1 cc. of the opalescent supernatant fluid 1 cc. of 0.2 per cent formalin (0.08 per cent formaldehyde) in saline was added and the mixture was allowed to stand in the ice box for 24 hours before using.

¹ The results of the series of intracranial reinoculations appear in Appendix B but not in the body of the paper because they did not yield information of value for the immunological classification. They are presented in graphic form in Appendix B and described briefly there in order to show that they do not furnish data contradictory to the pattern of the neutralization tests.

Live virus was given in saline as a 10 per cent suspension prepared within 2 hours of inoculation.

In each instance 2 cc. of the 10 per cent suspension (either formalinized or live) were inoculated in 10 piqûres into the shaved abdominal skin of *rhesus* monkeys. Each animal being immunized received 2 such doses at an interval of 2 weeks. Samples of blood to be used for testing for the presence or absence of neutralizing substances were taken prior to the 1st vaccinating dose and 2 weeks after the 2nd dose.

Description of Strains.—6 strains of the virus of poliomyelitis were used. In describing them we realize that the respective virulence of each does not represent a fixed quantity and that no satisfactory standards of virulence in experimental poliomyelitis have been set up. When certain of our strains had not been passed for several months, their virulence diminished and sometimes massive doses were necessary to recover them.

McC. Strain, Los Angeles, California, 1934.—This "human"² strain was recovered from the nasopharynx of a child during a mild abortive attack of poliomyelitis; the isolation and first few passages have been described (13). Material used for vaccination and subsequent intracerebral testing of the vaccinated monkeys came from a single monkey (C 6-7, Dec. 3, 1934)³ and represented the 3rd passage of the strain. The dose (for intracerebral inoculation) was 0.5 cc. of a 10 per cent suspension. The experimental disease produced by this and subsequent passages was definite, though not severe. The incubation period was from 3 to 5 days, the febrile period from 3 to 6 days and the animals seldom developed complete paralysis of all 4 limbs. A few attempts to infect monkeys by the nasal and cutaneous routes proved unsuccessful.

During the interim between preparing sera and performing the neutralization tests this strain died out, and recourse was had to another line of passage which had been carried on in another laboratory.⁴

Eventually 8th passage material (monkey 4-44, Mar. 23, 1936) was used in the neutralization tests. In respect to incubation period and type of experimental disease produced, this passage behaved the same as the earlier ones. The dose employed consisted in equal parts of (a) a 10 per cent virus suspension, and (b) serum to be tested. The mixtures were held for 2 hours in the incubator (37°C.) and inoculated intracerebrally in 0.5 cc. amounts.

² There is no standard definition of "human" and "passage" in experimental poliomyelitis. We have taken human to refer to strains not far removed from man and which usually do not lead to death of the monkey; passage to designate those strains which are well established in the monkey and which usually give rise to fatal experimental infection.

³ In this and similar notations the date, Dec. 3, 1934, refers to the day the cord was barvested.

⁴ We are indebted to Dr. Leslie T. Webster of The Rockefeller Institute for Medical Research for this material.

Wfd. Strain, Los Angeles, California, 1934.—This human strain was recovered from the medulla and spinal cord of a girl dead on the 6th day of poliomyelitis. Material for vaccination represented the 3rd passage (monkey C 5-5, Nov. 10, 1934). Material for subsequent intracerebral testing of vaccinated monkeys represented the 3rd passage (monkey C 5-5), the 4th passage (monkey C 1-03, July 28, 1935) and the 5th passage (monkey C 1-08, Aug. 14, 1935). Mention (2) has been made of the high intracutaneous infectivity of this strain; on subsequent passage it has produced experimental poliomyelitis more consistently when injected intracutaneously than has been noted with any of our other strains. The dosage used for intracerebral inoculation and the type of experimental disease produced were the same as with the McC. strain.

For neutralization tests 6th passage material was used (monkey C 1-20, Nov. 24, 1935). The dose was the same as in the neutralization test with McC. strain.

We. Strain, New Haven, Connecticut, 1931.—This human strain was recovered from the nasopharynx of a child suffering from a mild abortive attack of poliomyelitis; the isolation (14) and early passages (15) have been described. Material for vaccination and subsequent intracerebral testing of vaccinated monkeys represented the 9th passage (monkey C 7-4, Dec. 11, 1934). The experimental disease produced by this strain generally has been mild and characterized by features which recall those of the McC. and Wfd. strains.

For neutralization tests, material from the 9th passage was used (monkey C 1-25, Dec. 14, 1935) in the same dose as with the McC. and Wfd. strains.

Flexner Strain, New York, 1931.—This "passage" strain was recovered from the medulla and spinal cord of a fatal human case in the epidemic of 1931 in New York.⁵ When used for vaccination the strain was in its 11th passage (monkey C 8-1, Jan. 24, 1935). Material for subsequent intracerebral testing of vaccinated monkeys represented the 11th passage (monkey C 8-1) and the 12th passage (monkey C 1-06, Aug. 4, 1936). The intracerebral test dose was 0.5 cc. of a 0.5 per cent suspension. During the early passages this strain had maintained an intracerebral virulence comparable to that of the We. strain and at that time was considered as a human strain (1, 15), but by the 10th passage the virulence of the Flexner strain became enhanced in that it generally gave rise to extensive paralysis and often ultimately to death of the infected animal.

For neutralization tests, 15th passage material was used (monkey 4-91, May 9, 1936). The dose consisted of 0.5 cc. of equal parts of (a) 1 per cent virus, and (b) serum to be tested (10 per cent virus used June 1 proved too strong).

Aycock Strain, Vermont, 1921.—This well known and virulent passage strain was received in 1931.⁶ It was recovered in the fall of 1921 from a case in Vermont and has been subjected to many more passages in the monkey than any of the

⁵ This strain was received by us in 1932 through the kindness of Dr. Simon Flexner of The Rockefeller Institute.

⁶ This strain was received through the kindness of Dr. W. L. Aycock of The Harvard Medical School.

strains mentioned above. The exact number of passages is not known, "perhaps a hundred" (16). The experimental disease which followed its use was severe and generally fatal and, in this respect, was similar to that produced by the Flexner and Park strains.

For vaccination, material from monkey C 8-2 (Jan. 29, 1935) was used. For subsequent intracerebral testing of vaccinated monkeys, material from C 8-2 and from the next passage (monkey C 1-07, July 29, 1935) was used in a dose of 0.5 cc. of a 0.5 per cent suspension.

For the neutralization tests material from another passage was used (monkey 4-89, May 7, 1936). The dose consisted of 0.5 cc. of equal parts of (a) 1 per cent virus suspension and (b) serum to be tested; as in the Flexner strain.

Park Strain.—This highly virulent passage strain⁷ was received in 1931 when it had already been subjected to many passages in the monkey. It is a mixed strain and so the date of its primary isolation and the actual number of passages is unknown. It caused extensive paralysis of almost all infected monkeys and there were very few survivors.

For vaccination, material from monkey C 8-0 (Jan. 22, 1935) was used. For subsequent testing of vaccinated monkeys, material from C 8-0 and from the next and the 2nd following passages (monkey C 1-05, July 30, 1935, and monkey C 1-21, Nov. 19, 1935) was used. The dose varied from 0.5 cc. of 10 per cent to 0.5 cc. of 0.1 per cent suspension. For the neutralization tests, material from a later passage was used (monkey 5-10, June 9, 1936). The dose consisted of 0.5 cc. of a mixture of equal parts of (a) 0.2 per cent virus and (b) the serum to be tested.

Comparative neutralization tests with human sera have been reported already wherein the We., Flexner, Aycock and Park strains were designated respectively, W, F, Aycock and M (1).

*Experimental Animals.*⁸—*Macacus rhesus* monkeys of 2 to 3 kilos were used throughout for the production and testing of the antisera. As is not unusual in experimental poliomyelitis certain animals were used more than once. As regards the propriety of doing this, it is pertinent to state that daily temperature readings were taken for 4 weeks from the day of inoculation and also that 2 observers independently exercised the monkeys daily for as long as the animals were in stock, which frequently covered a period of months. It seemed unlikely, therefore, that a mild attack of the experimental disease would pass unnoticed. However, this did happen to us once before (1)⁹ and apparently happened again in the case of monkeys 4-71 and 4-93 of this series (Appendix A). Accordingly special mention of monkeys used more than once appears in Appendix A, in a footnote.

Preservation of Material.—The spinal cords and brain stems bearing the stock

⁷ This strain was obtained through the kindness of Dr. W. H. Park and Dr. E. R. Weyer, Bureau of Laboratories, Department of Health, City of New York.

⁸ All operations and inoculations were done under full ether anesthesia.

⁹ Paul and Trask (1), page 461.

strains were kept in 30 cc. of 50 per cent glycerine in 2 ounce soft glass bottles, at first stoppered with rubber, but later during the period of the neutralization tests, with aluminum screw caps. In the first 9 months of this study Merck's blue label glycerine made up in saline was used; in the second 9 months this was replaced by Kahlbaum's glycerine diluted to 50 per cent in distilled water. The stocks of virus and serum were kept in a refrigerator held between 2° and 4°C. Serum samples were kept without preservative in small glass bottles capped with "no-air" rubber stoppers.

Neutralization Tests.—As already stated, samples of serum were obtained from all vaccinated monkeys prior to the administration of the 1st vaccinating dose and 2 weeks after the 2nd. Neutralization tests were run on the postvaccinal samples of serum with the 6 stock strains of virus. Ordinarily, for each strain, we used 1 postvaccinal serum sample selected from a monkey vaccinated with live virus. As the work progressed and larger animal quarters were available, we included 2 postvaccinal (live and formalinized) samples of serum for each strain.

The technique employed was similar to that which we have previously described (1). It may suffice to say that the 6 strains of virus were used in the doses mentioned above. Samples of cord and brain stem from a single monkey were weighed, ground in sand with the appropriate volume of saline solution and the suspension was centrifuged in the angle centrifuge at 1500 R.P.M. for 5 minutes. The supernatant fluid was mixed with equal parts of undiluted monkey serum. The mixture was placed in an incubator at 37°C. for 2 hours and monkeys were subsequently inoculated intracerebrally with 0.5 cc. amounts. Daily temperature readings were made for a period of 4 weeks and all animals were exercised daily as long as they remained in the laboratory.

In each series of tests 2 controls were included, a protected control in which pooled human convalescent serum (collected October, 1931) was mixed with the virus, and an unprotected control in which normal monkey serum (representing the prevaccinal bleeding of 1 monkey) was mixed with the virus. The normal monkey serum chosen, represented the first bleeding from an animal which later was vaccinated with the strain of virus to be used in the current test. Some attempt at titration of the virus in decimals of the test dose was included in 7 of the 8 tests. The diluted virus was set up with equal volumes of the normal monkey serum.

In the experiment of Jan. 17 with the We. strain, where the results were seriously clouded by the occurrence of bacillary dysentery and again with the experiment of June 1 with the Flexner strain, when the protected control came down, the series of tests was repeated.

The results of our neutralization tests have been expressed as follows: + signifies that neutralizing properties were present in the serum, in that the test animal did not develop the experimental disease within the 4 week period of observation; — signifies that neutralizing properties were absent, in that the animal developed the experimental disease within an incubation period not more than 5 days longer than that of the unprotected control. If the incubation

period was longer than this, the result was recorded by \pm to signify partial neutralization. Thus the results were generally easy to classify but there was one exception; in the experiment of June 1. Monkey 4-71 developed fever without other signs of experimental poliomyelitis and subsequently resisted another intracerebral inoculation of active virus. In Appendix A this is shown as possible experimental poliomyelitis.

RESULTS

Comparative Neutralization Tests.—In Table I and in the appendices (where more details appear) the results of the neutralization tests are presented. They are arranged so that the strains appear in 3 groups which are designated Old, 1931 and 1934. The Old group includes the Park and Aycock strains. The 1931 (eastern) group includes the Flexner and We. strains and the 1934 (western) group includes the Wfd. and McC. strains. By noting the results listed under the experiments of May 11 and July 13, it may be seen that the Aycock and Park strains were compared by means of 8 pairs of tests; in 5 pairs the results were identical and in 3 pairs they were somewhat different, but not completely so ($2; \pm$ vs. $+$; $1; \pm$ vs. $-$). In general, therefore, the Old strains resembled each other. This statement is fortified by inspection of the table which reveals that the Old strains were more closely related to each other than to any of the 4 other strains. Further, it may be noticed that the Old strains were well neutralized by the homologous and 1934 antisera. In fact the Old strains were more frequently neutralized than were those of the other groups. This is worth noting because both the Aycock and Park strains were especially virulent for monkeys and the effective neutralization shown here suggests that immunological differences between the groups rest on a qualitative immunological basis.

The 2, 1931 strains were compared by 6 pairs of tests shown in Table I. The results were identical in 4 pairs, fairly close in 1 pair and dissimilar in 1 pair. Again, however, inspection of the table shows that the outstanding feature is that the 1931 strains appear more closely related to each other than to any other strains. That this relationship was independent of the mere virulence of the strains is indicated in Appendix A, where the titrations show that the Flexner was considerably more virulent than the We. strain. In support of this is the fact that when the Flexner strain was used as

5 per cent virus on June 1, the protected control and all monkeys except 4-71 came down with experimental poliomyelitis.

The 2, 1934 strains had 6 pairs of tests in common. The results were the same in each pair of tests and as far as these experiments are

TABLE I
Results of Neutralization Tests

Immune sera Monkeys vaccinated with	Vaccine	Vaccinated monkeys	Strains of virus					
			Old		1931		1934	
			Aycock	Park	Flexner	We.	Wfd.	McC.
			May 11	July 13	June 19	Jan. 17; Feb. 21	Jan. 9	Apr. 22
Old	Aycock	C 8-7 F.	+	+	+			
	C 8-2	C 9-8 L.	±	+	-	+	+	+
	Park	C 6-2 F.		+	-			
	C 8-0	C 9-4 L.	+	+	-	-	-	-
1931	Flexner	C 8-8 F.		-	-			
	C 8-1	C 9-9 L.	-	-	+	+	-	-
	We.	C 8-4 F.		-	-			
	C 7-4	C 9-3 L.	±	-	-	-*	-	-
1934	Wfd.	C 8-9 F.	+	+	-		+	+
	C 5-5	C 1-01 L.	+	+	-	-	+	
	McC.	C 8-3 F.		-	-			
	C 6-7	C 9-2 L.	±	+	-	-†	+	+

F., formalinized vaccine; L., live vaccine.

-, no protection.

+, protection.

±, partial protection.

+* -*, test repeated, result confirmed.

-†, test repeated, result first -, second ±.

} Results of neutralization tests.

concerned the 2, 1934 strains may be considered immunologically identical.

Table I also shows the immunological interrelationship of the groups. It can be seen that, in respect to the Old antisera, the 1931 and 1934 groups behaved similarly, but that they differed in their own cross neutralization tests. Furthermore, these groups appear com-

pletely different if the results with the We. sera are eliminated from consideration. This seems a fair thing to do because the We. serum in 2 tests gave no protection against the homologous strains.¹⁰ Therefore in this connection it should be regarded as normal monkey serum.

The 1931 and Old groups were related by the results with the good Aycock and the ineffective We. antisera and separated by the results with the Park, Flexner, Wfd. and McC. antisera. The 1934 group and Old group were related by all antisera except the Park. Thus the 1934 strains were found to be more closely related immunologically to the Old than were the 1931 strains. There is an added interest in this relationship because Kessel, Van Wart, Fisk and Stimpert (17) reported that a California strain isolated in 1935 was related, with merely minor immunological variations, to the well known M.V. virus of The Rockefeller Institute. It is to be noted that the Flexner strain we used was not the well known M.V. virus, which is a passage strain of many years standing, whereas ours was of relatively recent origin. Thus our results, although not exactly comparable, are not in disagreement with those of Kessel *et al.* (17).

Comparison of the Strains as Effective Antigens.—It can be seen in Table I that some antisera gave rise to protection in most of the tests, whereas some other antisera were generally ineffective. Thus the Aycock antisera were good whether prepared by live or formalinized vaccine. Contrariwise the We. antisera were poor after either method of preparation. The Wfd. and McC. strains were moderately broad, while the Flexner and Park strains were narrow in their antigenicity. Naturally these apparent differences in antigenic efficiency could depend entirely on the individuality of the vaccinated monkeys, but where the results with the live and formalinized vaccines were mutually corroborative the outcome was likely attributable to the virus. Thus it seems probable that the Aycock strain was an unusually effective antigen. This recalls the report of Flexner and Amoss (18) and of Rhoads (19) on special immunizing strains of the virus.

¹⁰ The partial protection in the test, on May 11, of the Aycock virus and We. antiserum was an inconsistency in the neutralization tests which is impossible to explain with the data at hand.

DISCUSSION

The discussion will be limited to the results of the cross neutralization tests, for they concern the major aims of the paper which are to measure and define differences between certain strains of the virus of poliomyelitis. Such differences as those of intracerebral or intranasal virulence have, of course, been long recognized, but it is a relatively recent (1, 11, 15, 17, 20-23) observation that so called immunological differences exist and it is upon these that we have laid special emphasis.¹¹ Whether these immunological properties of the virus of poliomyelitis are actually more fundamental than other measurable properties is a question we cannot answer, but in this report they have been selected for purposes of strain differentiation and classification. In this respect we believe our results are informative, not only in the differentiation and classification of strains but also on the nature of the differences between them. Most prominent is the fact that, on the basis of these reactions, those strains which have been subjected to the greatest number of monkey passages and which we had termed passage strains (1), do not stand in sharp contrast to groups of more recently isolated strains which we had termed human strains. There is no evidence in these studies to show that the immunological make-up of the strains has been warped, so to speak, by adaptation to the host. Similarities seem to exist between strains isolated from the same epidemic rather than between those which had about the same number of passages. That is, the immunological reactions tend to group together our 2 strains isolated from the eastern epidemic of 1931 in contrast to the 2 strains isolated from the western epidemic of 1934, which also fall together. Furthermore, there is no indication that the immunological reactions of our 2 strains of 1931 (which have had more passages in the monkey than those isolated in 1934) more nearly simulate those of the older strains (Park and Aycock) which have had most passages of all. This is

¹¹ Recently, Dr. B. F. Howitt (*Science*, 1937, 85, 268), commenting on various differences between strains of the virus of poliomyelitis, stated that she had recovered a peculiar strain from the epidemic in Sacramento, California, during the winter of 1934. The new strain differed immunologically from one isolated in the summer of 1934 in San Francisco and also differed from her passage strain obtained from the New York City Board of Health.

contrary to a suggestion we once made when we had worked with but one pair of human strains (1).

There seems little doubt that some epidemiological application is to be found in the immunological differences. In fact, significant results have been obtained in neutralization tests in which multiple strains were used with human sera (1, 15, 23). Consequently, it would seem that the present conception of the relation of human immunity to the epidemiology of poliomyelitis, which has been constructed on the basis of neutralization tests performed with human sera and but one strain of the virus, will have to be modified in the light of these newer results. In this connection it is of some interest that our sample of pooled convalescent sera, obtained from adults during the 1931 epidemic in New England, neutralized all of our 6 strains. It should be noted again that the convalescent sera were collected from adults, but this brings up many questions too numerous and too complex to warrant discussion here. However, one point is obvious: that, in an effort to clarify the subject, the extent to which antibodies specific for a local strain are present in a given population should be contrasted with the presence of antibodies for a foreign strain, and *vice versa*. It would seem more than probable that there would be some overlapping of these antibodies, but such an experiment would also probably help towards explaining some of the paradoxes now existent in the subject of the relation of neutralizing antibodies to clinical and subclinical immunity to poliomyelitis.

CONCLUSION

Qualitative immunological differences exist between early passage strains or so called human strains of the virus of poliomyelitis.

These differences show a relationship to the epidemic source of the virus and are exemplified in this study by 4 strains isolated in different years during an eastern and western epidemic of poliomyelitis.

APPENDIX A

Protection Tests Done Jan. 9 to July 13, 1936, with Six Strains of Virus of Poliomyelitis and Their Antisera

Vaccinated monkey	Serum samples	Date of bleedings	Strain and passage	Old		1931				1934	
				Aycock	Park	Flexner	We.		Wld.	McC.	
				4-89 nIV	5-10 nVIII		4-91 XV	C 1-25 IX			
		1935		0.5% virus\$ May 11	0.1% virus July 13	5% virus June 1	0.5% virus June 19	5% virus Jan. 17	5% virus Jan. 9	5% virus Apr. 22	
C 8-7 F.	1	Feb. 27	Aycock C 8-2 mIII	4-95 +	5-34 +	*4-82 - 4.8.K.26	*4-93 +				
	2	Mar. 28		4-98 - 4.5.D.6 4-94 ± 19.22.K.25	5-35 +	*4-75 - 11.12.K.34	5-13 - 6.8.K.15	4-14 0 Dys.D.9	4-08 +	4-73 +	
*C 6-2 F.	1	Jan. 30	Park C 8-0 mIII		5-36 +		*4-95 - 8.11.K.20				
	2	Feb. 28			5-50 - 7.10.D.21						
†C 9-4 L.	1	Mar. 27		4-93 +	5-37 +	*4-83 - 2.7.R.	5-14 - 4.6.K.18	4-11 0 Dys.D.9	4-01 - 6.12.K.21	4-76 - 6.11.R.	
	2	Apr. 24									
C 8-8 F.	1	Feb. 28	Flexner C 8-1 XI			5-01 - 6.9.D.68	5-15 - 12.14.D.14				
	2	Mar. 28			5-38 - 6.9.D.44	5-05 - 5.12.D.24					
†C 9-9 L.	1	June 26				*4-64 - 8.9.R.					
	2	July 24		*4-59 - 8.8.K.17	5-39 - 9.11.D.16	5-04 - 11.13.D.63	5-16 +	4-12 +	4-04 - 6.10.K.10	4-78 - 5.9.K.14	

C 8-4 F.	1	Feb. 6	We. C 7-4 IX	5-40 - 6.8.D.8	5-08 - 7.8.K.14	5-26 - 4.8.R.	4-16 - 5.9.K.9 4-10 - 4.10.R.	4-38 - 7.10.D.18 *4-15 - 6.12.K.18	4-02 - 5.12.R.	4-77 - 4.8.R.
	2	Mar. 7								
C 9-3 L.	1	Mar. 27	*4-58 ± 12.13.D.16	5-41 - 4.10.R.	5-06 - 5.7.K.34	5-27 - 6.8.D.15			*1-1 - 6.11.R. 4-05 +	4-74 +
	2	Apr. 23								
†C 8-9 F.	1	Mar. 14	4-97 +	5-44 +	*4-70 - 4.8.K.21	5-17 - 9.9.K.19			4-07 +	
	2	Apr. 11								
†C 1-01 L.	1	July 10	4-96 +	5-45 +	*4-71 ?? 2.R.	5-18 - 7.10.K.19	4-13 - 5.11.R.	4-33 0 Dys.D.19		
	2	Aug. 7								
C 8-3 F.	1	Feb. 6	McC. *C 6-7 III	5-46 - 9.10.D.11	*4-74 - 7.9.K.16	*4-86 - 7.9.D.17			4-03 +	4-79 - 5.10.R. 4-75 +
	2	Mar. 7								
†C 9-2 L.	1	Mar. 27	*4-50 ± 27.28.D.28	5-47 +	*4-73 - 7.14.K.15	5-19 - 7.9.D.65	4-09 - 7.9.R.	*4-18 ± 16.21.R.		
	2	Apr. 23								

D., died; K., killed; R., recovered; Dys., dysentery; Tbc., tuberculosis; R.W., remained well. III, 3rd passage; nIII, 3rd passage at our hands, etc.
+, protection; ±, partial protection; -, no protection; 0, monkey lost; ?, irregular fever; ??, possible experimental poliomyelitis.

Example. 4-98 -
4.5.D.6

Monkey 4-98, no protection. Fever 4th day. Paralysis 5th day. Died 6th day.

§ Note that final dilutions of virus are given.

* Previously used in experimental poliomyelitis.

† Previously used in experimental measles.

APPENDIX A—(Concluded)

	Old		1931				1934	
	Aycock	Park	Flexner	We.		Wid.	McC.	
Pooled human convalescent serum Oct. 23, 1931	4-89 nIV	5-10 nVIII	4-91 XV			C 1-20 VI	4-44 VIII	
	0.5% virus\$ May 11	0.1% virus July 13	5% virus June 1	0.5% virus June 19	5% virus Jan. 17	5% virus Jan. 9	5% virus Apr. 22	
	4-99+	5-49+	5-03± 15.18.D.64	5-20+	4-15+	4-06+	4-82+	
	4-67 8.10.D.15 4-08 8.9.D.9	5-51 13.14.R.	5-02 6.6.K.16	5-21 11.13.K.53	4-22 0 Dys.D.18		4-80 7.10.K.15	
Titration: Decimal test doses, 0.5 cc. intracerebrally:	4-63 5.11.K.18 4-64R.W.	5-48R.W. 5-53R.W.	4-32 6.9.R. 4-39 6.9.K.11	5-22R.W. 5-23R.W. ?	4-21R.W.			
		5-52R.W.		5-24R.W.				
1/1000								

Further Notes on Certain Monkeys Shown in Appendix A

In the case of the We., Flexner, Aycock and Park strains, fresh monkeys were used in the entire series of passages which furnished stocks of virus; with the McC. and Wfd. strains, some monkeys had been used before. For the McC. strain, monkey C 6-7 having failed to respond to the intranasal instillation of McC. virus on Oct. 23, 1934, received on Nov. 21, 1934, the same strain intracerebrally, became paralyzed and died Dec. 3, 1934, when the cord was harvested. For the Wfd. strain, monkey C 5-5 had failed to sicken after the intracerebral inoculation on Aug. 22 and Sept. 26, of non-infective material collected July, 1934, in California. On Oct. 30, 1934, the Wfd. strain was inoculated intracranially and on Nov. 10 C 5-5 was prostrate, killed with ether and the cord harvested.

Five of the 12 monkeys vaccinated for the purpose of producing antisera had previously been used in a study in experimental measles. Of these 12 monkeys, C 6-2 (vaccinated with formalinized Park virus), was the only one which previously had received material connected with poliomyelitis. This monkey had failed twice to respond to intracerebral inoculations of preserved monkey cord, presumably non-infective, from the 1931 and 1934 epidemics. Serum of C 6-2 was used in 2 tests.

In the 83 neutralization tests of the various sera (exclusive of titrations) we employed 65 fresh monkeys and 18 monkeys which had previously resisted the inoculation of serum virus mixtures, presumably non-infective. Of the 18 monkeys, 14 proved to be satisfactory because they developed the typical experimental disease in the tests; 2, 4-18 and 4-50, were probably satisfactory because they developed the experimental disease, but only after a prolonged incubation period, and 2, 4-71 and 4-93, proved that they had been unsatisfactory test animals by resisting subsequent intracerebral inoculation with active virus although they had remained free of symptoms during the neutralization test. However, this does not seriously affect the general results, since the experiment including 4-71 was discarded because the protected control came down, and since the result with 4-93 could be eliminated without affecting the direct comparison of the 1931 and 1934 groups of virus.

Histological examination of all monkeys furnishing the stocks of virus revealed the typical lesion of poliomyelitis in the medulla and in the cervical, dorsal and lumbar levels of the spinal cord. This was true also of all monkeys shown in the appendix in so far as 21 of them were examined histologically after experimental poliomyelitis.

APPENDIX B

Vaccination and Reinoculation of Monkeys with Homologous and Heterologous Strains

Strain and source of vaccine	Vaccinated monkeys	Week of experiment											
		1	5	10	15	20	25	30	35	40	45	50	
1 McC. C 6-7	C 7-8 F. C 8-3 F. C 9-2 L.	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓	1 P. ↓ ↓	2 ↓ ↓	3 P. 2 ↓	4 4 4	5 6 5	6 5			
2 Wfld. C 5-5	C 8-6 F. C 8-9 F. C 9-7 L. C 1-01 L.	↓ ↓ ↓ ↓	↓ ↓ ↓ ↓	↓ ↓ ↓ ↓	2 ↓ ↓ ↓	3 1 ↓ ↓	4 P. 4 ↓ ↓	6 ↓ ↓ ↓	5 2 ↓ ↓	6 4 ↓ ↓	6 6 6		
3 We. C 7-4	C 8-4 F. C 9-3 L.	↓ ↓	↓ ↓	↓ ↓	3 P. ↓	1 3 P.	2 4	4 1	6 P. 2	6 5	6 6		
4 Flexner C 8-1	C 6-4 F. C 8-8 F. C 9-9 L.	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓	3 ↓ ↓	1 P. ↓ ↓	2 ↓ ↓	5 6 ↓	6 6	6		
5 Aycock C 8-2	C 8-7 F. C 9-8 L.	↓ ↓	↓ ↓	↓ ↓	5 ↓	6 ↓	4 P. ↓	5 P. ↓	2 6 ↓	4 ↓	6		
6 Park C 8-0	C 6-2 F. C 8-5 F. C 9-4 L.	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓	5 ↓ ↓	2 ↓ ↓	1 ↓ ↓	4 ↓ ↓	2 ↓ ↓			

↓ ↓, vaccinations; F., formalized vaccine; L., live vaccine. First week of experiment began Jan. 15, 1935.

P., experimental poliomyelitis from vaccination. 1, intracerebral inoculation with strain 1, etc., animal remained well. 1P., intracerebral inoculation with strain 1, etc., animal developed experimental poliomyelitis.

Homologous protective bodies: present +, partial ±, absent —.

Explanatory Note for Appendix B

Appendix B illustrates the series of vaccinations and intracerebral reinoculations. It can be seen that 3 vaccinated monkeys became paralyzed as a result of the mere vaccinations and that the monkeys in general were not solidly immunized by intracutaneous vaccination. Thus, in the course of the intracerebral tests 12 of 14 vaccinated monkeys and 1 of 3 vaccinated and infected monkeys developed experimental poliomyelitis with paralysis. In 8 instances this followed tests with strains homologous to the vaccine and in 6 instances followed strains heterologous to the vaccine. The totals do not tally because C 8-4 became paralyzed twice; once after the homologous strain and 5 months later, after a heterologous strain.

It may be noted further, that of 5 vaccinated and 2 vaccinated and infected monkeys which proved to be immune to their homologous strain, 6 were also immune to the other strain of their own group (*i.e.* Old; 1931 and 1934; groups established on the basis of the cross neutralization tests). Of these 6 group immune monkeys, 4 subsequently were found to be susceptible to a strain of one of the other 2 groups. Therefore these results seem to substantiate the immunological classification described in the text, but their value is nullified because of the lack of controls for the interval of time which elapsed between vaccination and infecting intracerebral inoculation.

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SPONTANEOUS ENCEPHALOMYELITIS OF MICE, A NEW VIRUS DISEASE

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Early in January, 1933, a young mouse with a flaccid paralysis of the hind legs was found among the stock mice of this laboratory. By the intracerebral inoculation of other mice with a suspension prepared from a portion of the brain and spinal cord of the paralyzed mouse, a similar condition was reproduced in normal animals. Histopathological studies of the brain of the original mouse showed a perivascular infiltration and scattered necrotic ganglion cells. In the spinal cord similar changes were observed, though to a much more marked degree. As mice are used extensively in the study of virus diseases, and spontaneous diseases of mice are of particular importance to workers in the virus field, it was decided that a thorough study of this disease might be of considerable value. A preliminary account of results obtained in the study of the virus which appears to be the etiological agent of this disease has already been published (1).

Incidence of the Disease

Since the discovery of the original paralyzed mouse, mice similarly affected have been found on numerous occasions among the stock mice. Five strains of the virus have been obtained from six mice found spontaneously infected. These five strains have all been propagated in series by the intracerebral inoculation of normal mice with a suspension of the brain and spinal cord from an infected mouse.

The majority of the spontaneously infected mice occurred in the Swiss strain purchased from several dealers. The infection, however, was not confined to this strain. No accurate statistics on the incidence of the natural occurrence of the disease are available, but in

general it appears to be very low, not more than one in about two thousand of purchased mice. Several mice became spontaneously affected in the laboratory during experiments with other infective agents. Mice found naturally infected have usually been young, most of them being approximately 6 or 7 weeks of age.

Strains of Virus Studied

Six attempts were made to transmit the infective agent from six spontaneously affected mice to normal animals. Five were successful, and the strains have all been maintained through serial mouse to mouse passages by means of intracerebral injection.¹ All strains with the exception of strain IV have approximately the same degree of virulence. Strain IV is comparatively avirulent, and as a rule only a relatively few mice inoculated with it become paralyzed. With this strain it also sometimes occurred that none of the mice became paralyzed. The virus was maintained, however, by choosing one or two of the inoculated mice at random, removing their brains and cords, and injecting normal mice with a suspension of these organs. In certain experiments, on the other hand, it seemed almost as virulent as the other strains. By continuous passage in mice, all strains seemed to become more virulent. Cross immunity studies with four of these strains indicated that they are immunologically related. Immunological characteristics of the other two strains were not determined.

Symptomatology

The cardinal symptom observed in naturally infected mice was the paralysis of the hind legs, which was of a flaccid type. Apart from this the mice seemed well. In mice injected intracerebrally, after an incubation period ranging from 7 to over 30 days, the first sign was a weakness of one of the limbs, often a fore limb. This weakness progressed rapidly into a paralysis, often first of a fore limb and later of both hind limbs. At times, however, the initial paralysis was observed in the hind limbs. The extent of paralysis was usually much more marked in the hind than in the fore limbs, frequently progressing to the extent that the animal was entirely without the use of its hind legs. Progression in these cases was then only possible by the use of the fore limbs. In some mice

¹ One of the sick mice from which the strain known as strain II was procured, was kindly given to us by Dr. W. A. Sawyer.

the progress of the disease was arrested spontaneously at this stage, and the animal gradually recovered from the paralysis which in most instances leads to death. Complete recovery was seldom observed, however, and then only in animals which showed a mild degree of paralysis. Mice that lived after severe paralysis showed emaciation of the hind legs and other deformities of these extremities. Throughout the entire course of the disease, the mice appeared normal except for the obvious paralysis. In those markedly paralyzed in the hind quarters, a constant dribbling of the urine was often observed. The tail seemed never to become paralyzed.

Influence of Age on Susceptibility

The susceptibility of groups of mice of the same strain but of different ages to intracerebral inoculation of the virus was tested on

TABLE I
Morbidity and Mortality in Mice of Various Ages Following Intranasal Instillation of Virus

Age of mice	No. of mice used	Mice developing paralysis		Average onset of paralysis; time after inoculation	Mortality		Average time of death after inoculation
		No.	Per cent		No.	Per cent	
<i>days</i>				<i>days</i>			<i>days</i>
54-59	34	25	73.5	17.2	20	59	23.5
46-48	29	20	69	15.6	26	90	19.1
26-29	26	11	42	17.8	24	92	18.1
6-8	20	2	10	13.0	20	100	12.7

numerous occasions. Invariably the younger mice were found to be more susceptible than the older. Almost all suckling mice died without any paralytic symptoms having been observed. With increasing age (Table I), the incidence of paralysis increases and the mortality decreases up to approximately 6 or 7 weeks of age. In mice older than this the incidence of paralysis again decreases and the incubation period becomes lengthened. In adult mice of various ages the difference in the morbidity rate is often insignificant, but any difference has always been in favor of the older mice being less susceptible. The difference between two groups of adult mice at times is shown only by the average incubation period being slightly longer in the older group. In adult mice the incubation period as

well as the morbidity rate gives much better indices of susceptibility than the time of death or the mortality rate. The age of the mice used in the majority of the experiments reported in this paper was from 6 to 10 weeks at the time of inoculation.

Intranasal Instillation of Virus

It was early found that mice could be infected by intranasal instillation of the virus. The number of mice which became paralyzed as a result of infection by this route was always much lower than the number becoming paralyzed after intracerebral inoculation with the same virus suspension. The percentage of mice developing paralytic symptoms following the intranasal instillation of virus varied considerably. In a large number of the experiments no mice became affected. The highest percentage of infection obtained by this route was 36. During the first twenty-one brain to brain passages, strain I produced the highest incidence of paralysis. In later passages, however, it very seldom produced paralysis when inoculated by the intranasal route. The comparatively avirulent strain IV, though injected by this route in several instances, produced paralysis in only one. The time of onset of symptoms following intranasal instillation of virus was always appreciably later than when mice were inoculated with the same material intracerebrally. Because of the low incidence of paralysis following intranasal instillation, experiments made to determine whether young mice were more susceptible than older ones when inoculated by this route were inconclusive.

Immunity Following Intranasal Instillation of Virus.—It was a matter of major interest to determine whether mice which had remained well after an intranasal instillation of virus developed immunity to a subsequent intracerebral injection.

Four groups of mice (Table II) were given intranasal instillation of virus—groups A and B with strain I, and groups C and D with strain II. Two additional groups, E and F, of the same age were included to serve as controls. 5 weeks afterwards, groups A, C, and E were inoculated intracerebrally with strain I, and groups B, D, and F with strain II. An additional group of mice, G, of the same strain but approximately 6 weeks younger, was inoculated intracerebrally with strain I.

As seen in Table II, the results indicate that previous intranasal instillation with strain I produced some degree of immunity to the

subsequent intracerebral injection of the same strain as well as to strain II. The immunity produced by intranasal instillation of strain II was comparatively weak to both strains, which in all probability is due to the fact that the suspension of strain I used for intranasal instillation was far more virulent or contained a higher concentration of virus than that of strain II. This presumption is substantiated by the fact that, following the intranasal instillation of virus, several mice which had received strain I became paralyzed, whereas all those that had received strain II remained well. The results of this experiment also indicate that there is some immunolog-

TABLE II

The Effect of an Intranasal Instillation of Virus on the Susceptibility of Mice to Subsequent Intracerebral Inoculation

Mouse group	Strain of virus given intranasally	Strain of virus given intracerebrally 5 wks. later	No. of mice in group	Mice developing paralysis		Mortality	
				No.	Per cent	No.	Per cent
A	Strain I	Strain I	19	9	47.5	7	36.8
B	Strain I	Strain II	19	9	47.5	4	21
C	Strain II	Strain I	21	14	66.6	2	9.5
D	Strain II	Strain II	23	17	74	11	48
E	Normal control	Strain I	24	21	87.5	12	50
F	Normal control	Strain II	23	20	87	15	65
G	Normal controls 6 wks. younger	Strain I	22	22	100	22	100

ical relationship between the two strains. The influence of the age of the mice on their susceptibility is shown by the fact that all the mice in group G became paralyzed and died, whereas 50 per cent of those in group E survived.

Twelve additional experiments along the same lines have been performed, and the following observations have been made. Control mice injected intranasally with suspensions of normal mouse brains and spinal cords do not develop immunity. The degree of immunity following intranasal instillation of the virus seems to depend upon the virulence of the strain used. Strain I has produced a much higher degree of immunity than strain IV when administered intranasally. In fact, it was found very difficult to produce immunity with strain IV by this route, and in only one of three experiments did the results

indicate that some immunity was produced with this strain. Total absence of immunity also was shown on one occasion in mice given strain I intranasally and tested afterwards with the same strain. From the results of nine experiments it was concluded that an intranasal instillation of the virus had produced a mild degree of immunity to a later intracerebral injection of the same or other strains. In this manner additional evidence was obtained to indicate that strains I, II, IV, and V were immunologically related.

As the control mice in these experiments were of the same strain and age, and were kept under the same conditions as the mice which received the intranasal instillation of the virus, it is apparent that the immunity in the latter group was greater than that which normally develops with age. In fact, in most of these experiments an additional group of control mice was used for the intracerebral immunity test. This control consisted of a group of mice, usually twenty-five in number, of the same strain but younger than those under experimentation. The results invariably showed that the degree of immunity was highest in the intranasally infected mice, lower in the normal controls of the same age, and lowest in the third group of normal younger mice of the same strain.

Whether the relative immunity which follows an intranasal instillation of the virus is accompanied by the development of neutralizing antibodies has not been determined. It must be emphasized that the degree of immunity produced by this means is rarely marked. As the test for the demonstration of neutralizing antibodies is still very much in the experimental stage, it did not seem at all promising to attempt to determine whether the sera of intranasally inoculated mice contained more neutralizing antibodies than the sera of normal controls of the same age.

Ultrafiltration Experiments with the Virus

Early in the study of this disease it was found that the virus passed readily through Seitz filters and all grades of Berkefeld filters. In these filtration experiments the virus suspension was always made in a diluent containing 10 per cent of normal monkey serum. Efforts were made to determine approximately the size of the virus particles by filtration through graded collodion membranes. A total of eleven experiments were made. The technique and the type of diluent were

essentially the same as used in the filtration of poliomyelitis virus (2). In Table III are summarized the results of the filtration experiments. The virus passed through a membrane with an average pore diameter of 60 μ with relative ease, but as the pore size of the membranes was reduced less virus was demonstrated in the filtrate. On two occasions the virus was demonstrated after passage through a 43 μ membrane but was never demonstrated in the filtrate of the 39 μ membrane, which is therefore taken as the filtration end-point. Applying the formula of Elford to this ultrafiltration result, the particle size of the virus of encephalomyelitis of mice would appear to be 13 to 19 μ , and it is therefore practically the same size as poliomyelitis virus (2, 3). The technical difficulties encountered in

TABLE III
Filtration of Virus through Graded Collodion Membranes

Average pore diameter of membranes μ	No. of tests made	No. of times filtrate infective	Total No. of mice inoculated with filtrates	Paralysis in mice inoculated with filtrates	
				No. paralyzed	Per cent paralyzed
60	2	2	22	18	82
55	7	7	63	27	43
50	17	10	184	35	19
43	7	2	65	3	4.6
39	9	0	78	0	0
35	8	0	69	0	0

the filtration of poliomyelitis virus have been pointed out. The same difficulties were encountered with the virus of encephalomyelitis of mice. In the discussion of the filtration results with the virus of poliomyelitis, it was pointed out that the virus was in all probability smaller than calculated, and in fact this assumption was substantiated by Elford, Galloway, and Perdrau (3). The same criticism applies in the case of the virus of mouse encephalomyelitis, and it seems highly probable that the virus particles are considerably smaller than the ultrafiltration results indicate.

Preservation of the Virus

The preservation of the virus in infective brains and spinal cords in 50 per cent glycerine in the refrigerator was found to be a very

satisfactory method. Virus preserved in this manner proved to be infective for at least 150 days without apparent loss of activity. Its preservation by the method of desiccation in the frozen state, which has been so satisfactory for yellow fever virus, has not proven successful.

Distribution of the Virus in the Mouse

The infectivity of the blood of paralyzed mice was tested on several occasions. No virus was ever demonstrated. In addition, the infectivity of the blood was tested on the 1st, 3rd, 6th, and 8th days after an intracerebral injection of virus. On no occasion was there virus present. Following an intracerebral injection of virus, the brain was infective immediately, and the spinal cord usually became infective within 24 hours. In one experiment the spinal cord was tested for the presence of virus in one mouse on the 3rd day and in another on the 8th after intracerebral inoculation, with a negative result. In paralyzed mice the spinal cord was always infective and contained the virus in greater concentration than the brain. The amount of virus present was never very great, as titrations showed that 1 in 1000 was the highest dilution capable of producing infection.

The spleen, kidney, adrenal, and liver proved to be non-infective on two occasions, once in a mouse in the early stages of paralysis, and once in one which had been paralyzed for 20 days. The sciatic nerve on one occasion proved to be infective; the infectivity of other organs has not been tested.

Persistence of the Virus in Mice

Several experiments were undertaken to determine how long the virus persisted in the central nervous system of mice following intracerebral injection of the virus. For this purpose the mice were divided into two series, one consisting of mice which had become paralyzed, and the other of those which had remained well after an intracerebral injection of the virus. At irregular intervals one mouse of each group was killed and the infectivity of the brain and spinal cord was tested by the intracerebral injection of an emulsion of each organ into a group of mice. The results with the paralyzed mice

were briefly as follows: The virus was demonstrated in every instance, with one exception, up to 1 year after inoculation, which was the longest period tested. Judging by the average incubation period as well as by the number of mice becoming infected, there was invariably more virus in the spinal cord than in the brain. On several occasions the brain proved to be non-infective though the spinal cord contained virus. In general, there seemed to be a steady but slow decrease in virus content in both the brain and the spinal cord with the passing of time, although this decrease was more marked in the former organ.

The experiments to determine the persistence of the virus in mice which had remained well were performed with the comparatively avirulent strain IV. Approximately 100 mice were inoculated intracerebrally. All the mice which became paralyzed were removed and those which remained well were segregated and kept under observation. At irregular intervals up to 62 days after inoculation the infectivity of the brain and spinal cord was tested as has been described above. Virus was demonstrated on five occasions. The longest time that virus persisted in the central nervous system in this experiment was 48 days. In later experiments, however, virus was shown to persist for as long as 163 days following injection.

Neutralizing Antibodies

Preliminary investigations showed that the serum of mice which had been paralyzed for some time and were consequently immune to an intracerebral injection of virus, had developed a moderate capacity to neutralize the virus. The technique of the test which was finally adopted is briefly as follows:

The serum to be tested was mixed with an equal volume of three or more dilutions of filtered virus. These mixtures, with a similar set containing normal serum, were incubated for 3 hours at 37.5°C., and each was then injected intracerebrally into a separate group of twelve mice. Incubation of the mixtures was considered essential, as no protective action of the immune serum could be demonstrated if the inoculations were made shortly after mixing. In one experiment the mixtures were incubated for 24 hours. The results, however, were not satisfactory as a considerable proportion of virus had become inactive. Mice inoculated with the mixtures were kept under observation for at least 30 days.

The amount of neutralization obtained was not great, but a difference between the serum from the immune mice and that from the normal mice was invariably manifest.

An attempt was made to determine whether the resistance to infection developing with age in mice was accompanied by development of neutralizing antibodies.

The mice were divided into two large groups of equal numbers. One group was kept as the control, and the other was injected intracerebrally on Mar. 12 with strain IV of the virus. Nearly all the inoculated mice became paralyzed. On Apr. 12 a number of the paralyzed mice were bled, and their sera were pooled and designated as serum A. On the same date a similar serum pool was prepared

TABLE IV
Results of Neutralization Test with Sera from Infected and Normal Mice

Serum pool	Date when obtained	Mice source of serum	No. of mice used in test	Percentage of mice paralyzed	Average time interval between injection and onset of paralysis	Percentage of mice which died	Average time interval between injection and death
					days		days
A	Apr. 12	Paralyzed	35	80	16.7	83	19.7
B	Apr. 12	Normal	31	84	12.7	74	16.3
C	July 12	Paralyzed	33	55	15.2	67	18.2
D	July 12	Normal	31	68	13.2	61	17.0

from the normal controls and called serum B. These two serum pools were sealed in test tubes and stored in the ice box. On July 12 two other pools of sera were prepared, one, serum C, from paralytic mice, and the other, serum D, from the controls. Neutralization tests were done with all four of the serum pools in one experiment. Three dilutions, 1 in 10, 1 in 100, and 1 in 1000, of the virus (strain I) were used. Equal amounts (0.5 cc.) of each serum were mixed with each dilution of the virus. The mixtures were incubated for 3 hours at 37.5°C., and twelve mice were inoculated intracerebrally with each mixture. The mice were kept under observation for 40 days. The results obtained are summarized in Table IV. The mice which died before the 7th day were excluded.

An analysis of the results shows that there was a significant difference between the normal and immune sera of the first pools (sera A and B) in their power to retard the onset of paralysis. This difference was less significant between the immune and the normal sera

of the second pools (sera C and D). With regard to the incidence of paralysis, the only significant difference between the sera taken after 1 month and the sera taken after 4 months was that the second immune pool (serum C) prevented paralysis in a greater number of mice than did the first immune pool, and the difference between the two normal pools in this respect was less evident. The mortality rate showed no significant difference, although there was a suggestion of a lower mortality with serum C than with serum A.

This experiment consequently does not settle the question of whether the relative immunity of older mice is accompanied by a development of neutralizing antibodies. The results show, however, that neutralizing antibodies are produced as a consequence of infection with the virus, and that for some time, at least, there is an increase of the antibodies manifested by the fact that the serum obtained 4 months after infection was more potent than that obtained 1 month after infection.

Relation of the Virus of Mouse Encephalomyelitis to the Virus of Human Poliomyelitis

Because of several points of similarity between the virus of poliomyelitis and the virus under study, several experiments were performed to determine whether there was a relationship between them. This became especially important as two of the strains of virus were obtained from mice which had been inoculated with the virus of human poliomyelitis. These two strains are referred to in this paper as strains II and IV.

Rhesus monkeys were inoculated intracerebrally or intracutaneously with suspensions of mouse brain containing the virus of mouse encephalomyelitis. In every instance the inoculated monkeys remained well, and when given an intracerebral injection of poliomyelitis virus later all proved to be susceptible. In all, eleven *rhesus* monkeys were inoculated with strains I and II. In addition three African green monkeys were inoculated, two with strain I and one with strain II; these also remained well.

Several neutralization tests were performed with the virus of encephalomyelitis and poliomyelitis immune monkey sera. For controls, serum obtained from two monkeys before they were infected with the virus of poliomyelitis was available. The results of these tests showed that there was no significant difference in the neutralizing action of the serum of a normal monkey and that of the same animal after it had become immune to the virus of poliomyelitis.

As mentioned above, it has been shown that mice could be rendered relatively immune to encephalomyelitis as a result of an intranasal instillation of the encephalomyelitis virus. It was considered of interest to determine whether mice could be immunized against encephalomyelitis by the intranasal instillation of poliomyelitis virus.

In the first experiment, twenty-five mice were given four intranasal instillations of poliomyelitis virus. One month after the last inoculation, these mice, as well as thirty normal mice of the same strain and age, were given an intracerebral injection of the virus of encephalomyelitis. As the results seemed to indicate that the previous instillation of poliomyelitis virus had conferred a slight degree of immunity to a subsequent injection of the virus of encephalomyelitis, the experiment was repeated on a larger scale. The results of the second experiment showed no significant difference between the immunized mice and the normal controls.

The results of all these experiments suggest that there is probably no relationship between the virus of poliomyelitis and the virus of mouse encephalomyelitis.

Immunity

Paralyzed mice, as far as could be determined, appeared to be solidly immune to a second intracerebral injection of virus. Virus was still present in the central nervous system at this time, as a rule. Whether this immunity is permanent and persists after the disappearance of virus from the paralyzed mice has not been determined. A large proportion of the mice which remained well after an intracerebral injection of the virus were immune to a subsequent intracerebral injection of the virus.

The degree of immunity produced by age or after intranasal instillation of the virus is in many instances insignificant and never approaches that produced by intracerebral inoculation of the virus. Mice inoculated intraperitoneally with a massive dose of the virus did not develop immunity to a subsequent intracerebral injection. The application of virus to the scarified skin did not lead to immunity, nor could immunity be demonstrated in normal mice placed in contact with mice infected either spontaneously or artificially by intracerebral or intranasal inoculation of virus. In these experiments, it must be emphasized that the control mice were of the same strain and

age and were kept under the same conditions as the experimental mice. The degree of immunity often produced by intranasal instillation of the virus was so slight that in order to obtain significant results large numbers of mice had to be used.

Epidemiology

The incidence of spontaneous paralysis is very low. There is apparently a natural immunity which develops with the increase of age. Following the intranasal instillation of virus, comparatively few mice develop paralysis. A relative immunity, however, is often produced. Furthermore, mice which had become spontaneously infected were almost invariably young. These facts would seem to indicate that the virus is widespread and that only unusually susceptible mice or possibly mice exposed to massive dosages of virus develop clinical signs of infection. Suckling mice under natural conditions might become infected and die without showing signs of paralysis. The majority of the mice appear to build up an immunity gradually. Contact experiments in which apparently normal mice were placed in the same cages with those infected either by intracerebral inoculation or by intranasal instillation have not produced evidence of infection as a result of the contact. Nor did these mice develop immunity as compared with the control mice which were kept in a similar environment but not in contact with infected animals. The addition of several mice found spontaneously infected to a cage of normal mice likewise failed to produce infection in the normal animals. In this experiment the mice were allowed to become overcrowded by breeding, and as more spontaneously infected mice became available, they also were added. The experiment was allowed to continue for several months. At its termination six mice were taken at random from this case and the infectivity of the spinal cords tested. No virus was demonstrated. The failure to produce infection or immunity by intraperitoneal injection of massive doses of virus, the absence of virus at any time in the blood, and the absolute neurotropism of the virus itself make it seem probable that blood-sucking insects are not the means of spreading the infection. Nevertheless, as our experimental mice at times were heavily infested with a species of mite, this arthro-

pod was tested for its capability of transmitting the infection, by placing normal mice in jars containing many mites obtained from infected mice. The results were entirely negative.

The presence of healthy carriers would help greatly to explain the ascertained facts. That they can be produced has been adequately demonstrated, but whether they are capable of giving off virus has not been determined.

Pathology

Macroscopically nothing abnormal has been observed. Microscopically there is a perivascular round celled infiltration throughout the central nervous system, which is particularly marked in the spinal cord. An acute necrosis of ganglion cells, especially marked in the anterior horn cells, is a striking feature. These necrotic changes have been observed before the onset of paralysis. The process is exceedingly acute, necrosis being soon followed by neuronophagia. The ganglion cells in other parts of the cord are not affected to the same extent as those of the anterior horn. The cells of the posterior root ganglia appear to remain unaffected.

Mice which have been paralyzed for several months show a relative decrease in the number of anterior horn cells. At this stage, virus can still be demonstrated in the spinal cord. Perivascular infiltration is present, although to a much less extent. No intranuclear inclusions have been observed in infected brains or spinal cords.

SUMMARY

1. The characteristics of a filterable virus obtained from mice found spontaneously paralyzed and showing lesions of encephalomyelitis are described.

2. The course of the disease in mice, following intracerebral inoculation, is briefly as follows: After an incubation period varying from 7 to over 30 days a flaccid paralysis of one of the limbs appears. This paralysis usually spreads rapidly until all four limbs are affected. Young mice are more susceptible than older ones, and very young mice, less than 4 weeks of age, usually die without showing signs of paralysis.

3. Adult mice often show no signs of infection after an intracerebral inoculation of virus. A number of these mice, although showing no signs of paralysis, nevertheless have become infected, a fact which is

demonstrated by recovery of the virus from the mice as well as by histopathological studies.

4. Intranasal instillation of the virus is the only other method of producing the infection. This method, however, produces paralysis in only a small percentage of the mice. Following intranasal instillation of the virus, there often develops a slight immunity to a subsequent intracerebral injection of virus.

5. The paralysis in the surviving mice recedes gradually, but a permanent residual paralysis, usually of the hind legs, is almost invariable. Such mice, however, are virus carriers, as the virus can be recovered from the spinal cord for 1 year after infection.

6. Paralyzed mice are immune to a subsequent intracerebral injection of the virus. There is evidence that neutralizing substances are present in the immune mice. A considerable proportion of the mice which have remained well after an intracerebral injection of virus are immune to a second injection.

7. The virus resists the action of 50 per cent glycerine at from 2-4°C. for at least 150 days. It passes all grades of Berkefeld filters with ease. By the use of graded collodion filters, the size of the virus particle has been determined to be probably about 13 to 19 $m\mu$.

8. The virus of mouse encephalomyelitis is not pathogenic for *rhesus* monkeys. No evidence of immunological relationship with the virus of human poliomyelitis has been obtained.

9. The anatomical basis of the paralysis is an acute necrosis of the ganglion cells of the anterior horn of the spinal cord. Isolated ganglion cells of the cerebrum also undergo necrosis. Following the acute necrosis of the ganglion cells, there is a marked neuronophagia. A perivascular infiltration is observed in the brain and spinal cord.

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STUDIES ON HAEMOPHILUS INFLUENZAE*

I. INFECTION OF MICE WITH MUCIN SUSPENSIONS OF THE ORGANISM

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Investigations of the immunologic problems concerned in infections with *Haemophilus influenzae*, the most important and serious of which is meningitis, have been hindered to a very considerable extent by the lack of an experimental animal with adequate susceptibility to infection by this organism. Studies dealing with the development of a specific form of treatment would be facilitated to a considerable extent if a true infection could be established in a laboratory animal.

Many investigators (1-3) in the past have attempted to produce infections in animals with *H. influenzae*. While it is possible to kill laboratory animals with sufficiently large doses of the organism, it cannot be said that a true infection, characterized by *in vivo* multiplication of organisms, has ever been demonstrated. It is likely that death has occurred in most instances simply from the toxic effects of such large doses. Various animals, including monkeys, rabbits, guinea pigs, and mice have been used in such studies and all react in a similar manner. It is obviously difficult to pursue experimental studies of such problems as active and passive immunization and the development of methods for standardizing and appraising the related biological products, under these circumstances.

Nungester, Wolf, and Jourdonais (4) first demonstrated the effect of mucin on the mouse virulence of the pneumococcus, streptococcus, and staphylococcus and later, Miller (5) found that mice, normally resistant, can be infected by mucin suspensions of the meningococcus. It seemed possible, therefore, that mucin might also be utilized to produce an infection in mice with the influenza bacillus.

The experiments¹ to be described in this communication show that:
(a) the suspension of *H. influenzae* in mucin enhances the power of this

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¹ All strains of *H. influenzae* used in these experiments were isolated from the cerebrospinal fluid of cases of meningitis, were smooth in character and serologically homogeneous in type (presumably the type b of Pittman).

organism to infect mice; (b) *in vivo* multiplication of the organism with subsequent death of the animal occurs; (c) repeated mouse passage appears to enhance the "mucin virulence;" and (d) protection tests and passive immunization experiments can be carried out with this method.

Experiments Which Establish the Occurrence of a True Infection in Mice

The evidence which appears to establish the occurrence of a true infection in mice with *H. influenzae* suspended in mucin was obtained by employing the following methods.

Mucin suspensions were prepared according to the techniques described by Miller (6) and more recently by Nungester, Jourdonais, and Wolf (7), using granular mucin, type 1701-W.² In all experiments, except the one otherwise designated, mucin prepared according to the method of Miller was used.

The organism was grown on peptic digest blood agar plates as described in a previous paper (8) for 10 to 18 hours at 37°C. Usually the seed inoculum was the heart blood of an infected mouse, evenly spread over the surface of the plate with a capillary spreader. The growth, which generally was confluent, was carefully scraped up in 1.5 cc. of sterile infusion broth, yielding a dense well suspended preparation. Broth was used for making suspensions and dilutions in order to avoid possible toxic effects of physiological salt solution. In addition to observing the plates carefully for contaminating colonies, purity of the suspension was determined by examining stained smears and subcultures on peptic digest-blood agar plates. Smears of these young cultures of virulent organisms exhibited no clumping and a minimum of pleomorphism.

In preparing mucin suspensions for mouse inoculation, the above broth suspension was diluted 1:4 with mucin. From this mixture further dilutions in mucin were carried on decimally using a separate sterile pipette for making and mixing adequately each dilution. For inoculation of control mice, as well as for plate counts, a similar series of dilutions was prepared in sterile infusion broth. An intraperitoneal dose of 1 cc. of the resulting mucin or broth suspensions was employed. In most instances, 6 mice were inoculated with each dilution: 4 received the suspension in mucin, and 2 the control suspension in broth. Such an experiment is illustrated in Table I.

It is apparent that mice receiving mucin suspensions of the organism succumbed to a much smaller infecting dose than did those receiving the corresponding broth suspension—in this case, the majority of mice inoculated with the 1-40,000 dilution in mucin died, whereas all control mice survived beyond a dilution of 1-4 in broth.

² Purchased from the Wilson Laboratories, Chicago, Illinois.

The character of the infection produced in mice was as follows:

Usually no symptoms were exhibited for several hours after inoculation. The animal then gradually developed evidences of a severe infection, *i.e.*, refusal of food, ruffling of fur, diarrhea, and lassitude. These symptoms increased progressively until death; they were not, however, specific in character. The majority of mice died between 18 and 36 hours, although occasional animals survived for as long as 72 hours. In all instances, mice dying even on the 3rd day showed positive cultures at autopsy. Mice living longer than 72 hours rarely succumbed to the infection. Histopathological studies have not yet been made to determine

TABLE I

The Effect on Mouse Virulence of Suspending H. influenzae in Mucin

Dilution of organisms*	Mucin suspension		Broth suspension	
	D	S	D	S
1-4	4	0	1	1
1-40	4	0	0	2
1-400	4	0	0	2
1-4,000	4	0	0	2
1-40,000	3	1	0	2
1-400,000	0	4	0	2
1-4,000,000	0	4	0	2
1-40,000,000	0	4	0	2

* *H. influenzae*, F. strain, isolated from the cerebrospinal fluid of a case of meningitis.

D, died.

S, survived.

the extent and character of any pathological processes occurring in the mice infected by this method.

At autopsy, the peritoneal exudate was variable in amount, seropurulent in character, and occasionally mucoid. Smears of such exudate usually exhibited large numbers of *H. influenzae* and many leucocytes, a preponderance of which were polymorphonuclear cells. When a virulent organism was used, very few bacteria were phagocytized. A greater degree of phagocytosis was observed following the inoculation of an avirulent organism. Depending on certain circumstances the organisms appeared as large swollen, globose bodies, as described by Pittman (3). A more detailed study of these forms will be reported in a subsequent paper.

Cultures of both heart blood and peritoneal exudate yielded extraordinarily

large numbers of organisms. They were so plentiful in the heart blood as to be easily seen by direct smear. Furthermore, the swollen forms mentioned above could be demonstrated in smears of the heart blood. Centrifugated or filtered peritoneal washings gave a positive precipitin test with immune serum, thus indicating the presence of free precipitinogen, presumably soluble specific substance. This reaction was demonstrable even after a considerable dilution, as is evident from Table II.

TABLE II

Precipitin Reactions with Peritoneal Washings from Mice Succumbing to H. influenzae Infection

Mouse No.	Dilution of peritoneal washings								Control
	Undiluted	1-2	1-4	1-8	1-16	1-32	1-64	1-128	
1	++	++	++	++	++	++	+	+	0
2	++	++	++	++	++	+	+	0	0
3	++	++	++	++	+	+	0	0	0
4	++	++	++	+	0	0	0	0	0
5	++	++	++	++	++	+	0	0	0

The peritoneal washing of each mouse was made up to a volume of 6 cc. with physiological salt solution, centrifugated at high speed, and the supernatant fluid diluted as indicated before layering over anti-*H. influenzae* horse serum. Readings were made after 30 minutes at room temperature.

TABLE III

Invasion of Blood Stream Following Intraperitoneal Injection of H. influenzae

Mouse No.	Amount of peritoneal washing injected with 1 cc. mucin	Colonies in 1 drop of tail blood			
		15 min.	30 min.	45 min.	60 min.
	cc.				
D 70	0.5	38	300±	+++	+++
D 71	0.25	2	147	121	+++
D 72	0.1	5	52	83	+++

Invasion of the blood stream with *H. influenzae*, as determined by plating a drop of tail blood at 15 minute intervals after inoculation, occurred with great rapidity following intraperitoneal injection of mucin suspensions of the organism. This is illustrated in Table III.

The data presented above constituted no evidence that multiplication of the organism occurred in the infected mouse, although such

appeared very probable. Positive evidence on this point would be obtained if direct mouse to mouse passage of the organism could be carried out for an appreciable number of transfers. In order to determine this, the following experiment was performed.

Normal mice were inoculated with a lethal dose of *H. influenzae* (strain 62) suspended in mucin. After death of the mice, either a small quantity of peritoneal washing (usually 0.1 cc. proved lethal) or 1 drop of heart blood was mixed with 1 cc. of mucin and injected intraperitoneally into a normal mouse. As soon as possible after death of this animal, the process was repeated. At the time of each

TABLE IV

*Comparison of the Effect of Two Different Mucin Preparations on the Virulence of H. influenzae for Mice**

Dilution of organisms†	Mucin suspension (Nungester)‡		Mucin suspension (Miller)‡		Broth suspension	
	D	S	D	S	D	S
1-4	4	0	4	0	4	0
1-40	4	0	4	0	2	2
1-400	4	0	4	0	0	4
1-4,000	4	0	4	0	0	4
1-40,000	4	0	2	2	0	4
1-400,000	3	1	2	2	0	4
1-4,000,000	1	3	2	2	0*	4
1-40,000,000	1	3	0	4	0	4

* Swiss albino strain of mice.

† *H. influenzae*, strain 62, 45th mouse passage, 11th direct mouse to mouse passage.

‡ Relative viscosity approximately 10, as determined by the Ostwald viscometer.

§ 5 per cent suspension of mucin.

mouse to mouse inoculation, control smears and cultures were made to prove that the inoculum had not been contaminated. In the first part of this experiment 9 successive mouse to mouse passages were obtained before temporary interruption by contamination. Later a similar series of 11 passages was carried out. It is inconceivable that this would have been possible without actual *in vivo* multiplication of the organism.

In one experiment only, infection of mice with *H. influenzae* suspended in mucin prepared according to the method of Nungester, Jourdonais, and Wolf was compared with that obtained by the use of Miller's method of preparation. From this experiment, illustrated in Table IV, it appears that the former type of suspen-

sion produces more uniform results. More detailed comparative studies are being made.

Summarizing the above experiments, it is evident that: (a) with the aid of mucin, fatal infections with *H. influenzae* can be produced in mice; (b) actual multiplication of the organism with invasion of the blood stream occurs during such infection; and (c) the lethal dose of *H. influenzae* for mice is greatly decreased by suspending the organism in mucin.

TABLE V
Variation in Susceptibility of Mouse Strains to H. influenzae

Dilution of organisms	Unknown stock				Albino Swiss strain				Black Swiss strain			
	Mucin		Broth control		Mucin		Broth control		Mucin		Broth control	
	D	S	D	S	D	S	D	S	D	S	D	S
1-4	7	0	3	1	8	0	4	0	8	0	4	0
1-40	7	0	3	1	8	0	4	0	8	0	4	0
1-400	5	2	1	3	8	0	1	3	8	0	0	4
1-4,000	4	3	0	4	8	0	1	3	7	1	0	4
1-40,000	1	6	0	4	4	0*	0	4	4	0*	0	4
1-400,000	0	7	0	4	5	3	0	4	1	7	0	4
1-4,000,000	0	7	0	4	1	7	0	4	0	8	0	4

* Only 4 mice were inoculated in these 2 groups.

The Importance of Standard Mouse Strains in Determinations of the Virulence of H. influenzae

Very irregular and spotted deaths were noted in our earlier experiments with stock mice purchased from various animal supply houses. Since the work of several investigators (9, 10) has established the value of inbred strains of mice for experiments of this type, two such strains were examined in the hope of finding one which would yield more consistent and uniform results.

Table V demonstrates the comparative virulence of *H. influenzae* for (a) a heterogeneous and unknown genetic stock purchased from dealers, (b) an albino Swiss strain, and (c) a black Swiss strain of mice.³ Breeding of the Swiss mouse strains was carried on in this

³ Both of the latter strains were obtained from the Army Medical School.

laboratory; all animals subsequently used for experimental purposes were descendant from an original 30 stock mice of each strain.

These results show that not only were the mice of both the albino and black Swiss strains more susceptible than those of unknown and heterogeneous stock, but also that a much sharper end-point was obtained with either of the two former strains. No significant difference was noted in susceptibility of the albino and black Swiss strains, as indicated by ultimate survival of the mice. It was observed, however, that the duration of the infection in the black mice was consistently longer, that is, such mice, although obviously sick, did not succumb in as short a period of time, possibly due to slightly greater resistance of this strain, or more probably because these mice were in general older and larger than the albino Swiss mice. Inasmuch as the black mice were also more vicious and difficult to breed, the albino strain was selected for use in the subsequent experiments.

It is, of course, impossible entirely to eliminate the occasional inconsistencies due to individual animal variation in experiments of this kind, but it is of great importance to reduce such inconsistencies to a minimum. That such can be accomplished to a considerable extent is evident from the above data obtained by the use of a standard, inbred mouse strain.

Increase in Mucin Virulence of H. influenzae by Repeated Passage through Mice

It is ordinarily difficult, if not impossible, to increase significantly the virulence of an organism for a species of animal naturally resistant to infection with that organism. That mice are normally rather resistant to *H. influenzae* is apparent from the fact that in broth suspensions large numbers of organisms are required to kill these animals. With the aid of mucin, however, a much smaller dose is lethal; the organism under this condition acquires, at least relatively, an increased virulence, which we shall term its mucin virulence for the purposes of this discussion. The following experiment was carried out to determine whether or not the mucin virulence of *H. influenzae* could be increased by repeated passage through mice, employing suspensions in mucin as the inoculum.

The strain of *H. influenzae* used, No. 62, was isolated from the cerebrospinal fluid of a case of influenza bacillus meningitis on Oct. 6, 1936. Typical smooth colonies were obtained. Supernatants of broth cultures gave a precipitin reaction with anti-*H. influenzae* horse serum in a dilution of 1-32. The virulence of this organism was determined in the manner described above, with organisms obtained from the first subculture after isolation. 45 mouse passages of this strain were carried out, the greater proportion of them being direct mouse to mouse transfer employing peritoneal washings or heart blood in mucin as the inoculum. During the occasional 2 or 3 days' interval between two series of passages, the heart's blood was cultured in whole defibrinated rabbit blood, incubated 12 to 18 hours, and stored in the ice box. Virulence tests were performed at intervals, the suspension being prepared from peptic digest blood agar plates inoculated with heart blood of the respective passage mouse and incubated for 10 hours. Bacterial counts were made in the following ways: (a) by plating 1 cc. of the control broth dilutions with 10 cc. of peptic digest blood agar and incubating 72 hours at 37°C., and (b) by carefully smearing 0.25 cc. of each dilution over the surface of a chocolate or peptic digest blood agar plate with a capillary spreader and incubating for 24 or 48 hours.

The results of seven virulence titrations are presented in Table VI.

It can be seen that after 10 passages, the mucin virulence of this organism for mice was increased approximately one hundred-fold, and that subsequent repeated mouse passage did not augment this virulence to any significant degree. On the other hand, the ability of the organism, without mucin, to kill mice was not increased.

Plate counts have indicated that the smallest number of bacteria required to kill all 4 mice varied from 340,000 organisms in the first titration to 7,800 organisms and 27,200 organisms after the 29th and 45th mouse passages, respectively. It is felt, however, that little reliance can be placed on these estimates in regard to the absolute number of organisms present, not only because of considerable differences obtained by the two methods of plate counts, but also because direct counts on a few of these suspensions have demonstrated 10 to 50 times as many bacteria as the viable counts have indicated. The fact that the conditions under which the suspensions have been prepared were kept as constant as possible, and also that the virulence titrations checked remarkably well, indicates that the minimum lethal dose is sufficiently large to be relatively unaffected in a decimal series of dilutions by moderate differences in the original suspensions.

A study of the comparative mucin virulence of a series of smooth

TABLE VI

Increase in Mucin Virulence of *H. influenzae* Strain 62 by Repeated Mouse Passage

Mouse passages.		0				4th				10th				17th				27th				29th				45th			
Dilution of organisms		Mucin		Broth control		Mucin		Broth control		Mucin		Broth control		Mucin		Broth control		Mucin		Broth control		Mucin		Broth control					
		D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S				
1-4		4	0	2	0	4	0	2	0	4	0	2	0	4	0	2	0	4	0	2	0	4	0	2	0				
1-40		4	0	2	0	4	0	1	1	4	0	2	4	4	0	2	4	4	0	2	4	4	0	2	4				
1-400		4	0	2	0	4	0	0	2	3	1	0	2	4	0	2	4	0	1	1	4	0	2	4	0				
1-4,000		2	2	0	2	4	0	0	2	4	0	2	4	0	0	2	4	0	0	2	4	0	2	4	0				
1-40,000		1	3	0	2	3	1	0	2	4	0	2	4	0	2	2	4	0	2	2	4	0	2	4	0				
1-400,000		1	3	0	2	1	3	0	2	3	1	0	2	1	3	0	2	2	2	2	2	2	2	2	0				
1-4,000,000		0	4	0	2	0	4	0	2	1	3	0	2	2	1	3	0	2	2	2	2	2	2	2	0				
1-40,000,000		0	4	0	2	0	4	0	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4				

TABLE VII

Comparative Mucin Virulence of a Series of Smooth Strains of *H. influenzae*

Dilution of organisms	Strain 1				Strain 3				Strain 4				Strain 5				Strain 14				Strain 15			
	Mucin		Broth control		Mucin		Broth control		Mucin		Broth control		Mucin		Broth control		Mucin		Broth control		Mucin		Broth control	
	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S
1-4	4	0	1	1	4	0	2	0	3	1	0	2	4	0	2	0	4	0	2	0	4	0	2	0
1-40	4	0	0	2	4	0	0	2	3	1	0	2	4	0	2	2	4	0	2	2	4	0	2	2
1-400	4	0	1	1	4	0	0	2	2	2	0	2	1	1	1	1	3	1	0	2	1	0	2	2
1-4,000	4	0	0	2	4	0	0	2	0	4	0	2	0	0	2	2	4	0	2	0	4	0	2	2
1-40,000	1	3	0	2	3	1	0	2	0	4	0	2	4	0	2	2	4	0	2	0	4	0	2	2
1-400,000	0	4	0	2	1	3	0	2	0	4	0	2	0	4	0	2	0	4	0	2	0	4	0	2
1-4,000,000	1	3	0	2	1	3	0	2	0	4	0	2	0	4	0	2	0	4	0	2	0	4	0	2

strains of *H. influenzae* has been made. These strains have been maintained for a variable period of time by weekly transplants in fresh, whole, defibrinated rabbit blood. During this period, all the cultures have retained their smooth characteristics, as evidenced by colony morphology, as well as the type of growth and production of specific precipitating substances in broth culture. The results of virulence titrations in Table VII show a difference in the mucin virulence of these organisms. Strains 1 and 3 possessed a mucin virulence comparing favorably with that of the F. strain (Table I) and almost equal to that of strain 62 after repeated mouse passage. On the other hand, strains 4, 5, 14, and 15 exhibited a lower mucin virulence, comparable to that of strain 62 before mouse passage. These differences were not correlated with the duration of the period of artificial cultivation in blood, since strains 1 and 3 were isolated before the other strains of lower virulence. It is of further interest that, regardless of the mucin-virulence, there was no essential difference in the ability of the organisms to kill mice without mucin.

It would appear, therefore, that although the mucin virulence of *H. influenzae* for mice can be increased by repeated mouse passage of the organism in mucin suspension, ultimate fixation of such virulence occurs, beyond which no further increase is possible by this method. Furthermore, smooth strains suspended in mucin vary considerably in mucin virulence as compared with that of such a fixed strain, without any significant difference in their ability to kill mice in broth suspension.

Passive Immunization of Mice

The establishment of a true infection with *H. influenzae* in mice is of importance because it provides a means of determining the value of passive and active immunization. The following preliminary experiments are presented to illustrate this point.

In the first experiment, mice were injected with 0.5 cc. of anti-*H. influenzae* horse serum⁴ administered intraperitoneally or intravenously 2 hours before

⁴ The anti-*H. influenzae* horse serum used in these tests was obtained from a horse which has been kept under continuous active immunization for a number of years. Immunization has been maintained by repeated series of injections of formalinized suspensions of the 24 hour growth of a smooth meningeal strain. The strain used for this purpose has been replaced frequently by a freshly isolated one of the same serologic type, presumably the type b of Pittman.

inoculation with the suspensions of the organism which had been isolated from the cerebrospinal fluid of a case of meningitis (Br.). Plate counts showed the

TABLE VIII
The Mouse Protective Action of Anti-H. influenzae Horse Serum

Dilution of organisms*	Immune serum				Controls			
	0.5 cc. Anti-H. influenzae serum, ip		0.5 cc. Anti-H. influenzae serum, iv		Mucin suspension of organisms		Broth suspension of organisms	
	D	S	D	S	D	S	D	S
1-4	3	0	3	0	3	0	1	0
1-40	0	3	0	3	3	0	1	0
1-400	0	3	0	3	3	0	0	1
1-4,000	0	3	0	3	1	2	0	1
1-40,000	0	3	0	3	1	2	0	1
1-400,000	0	3	0	3	0	3	0	1

ip = intraperitoneal administration.

iv = intravenous administration.

* *H. influenzae*, Br. strain.

TABLE IX
The Mouse Protective Action of Anti-H. influenzae Horse Serum against a Lethal Test Dose of H. influenzae in Mucin

Dilution of serum	Anti-H. influenzae horse serum 0.5 cc. ip		Normal horse serum 0.5 cc. ip	
	D	S	D	S
Undiluted	1	5	6	0
1-4	0	6	6	0
1-16	1	5	5	1
1-64	0	6	6	0
1-256	3	3	6	0
1-1,024	5	1	4	2

All mice received 1.0 cc. intraperitoneally of 1-4,000 mucin suspension of *H. influenzae*, strain 62, 17th mouse passage. 4 additional control mice succumbed to the test dose of organisms.

original suspension, prepared in the usual manner, to contain 6,000,000,000 organisms per cc. The results, shown in Table VIII, demonstrate obvious protection.

In a second experiment, the protective action of anti-*H. influenzae* horse serum was titrated against a standard dose of organisms suspended in mucin. The organism employed was strain 62 after 17 mouse passages, suspended as described above. The virulence of the organism in this suspension (Table VI, 17th mouse passage) was such that 1 cc. of a 1-4,000 dilution killed all of 4 mice inoculated, and 2 of 4 mice succumbed with 1 cc. of the 1-40,000 dilution. Plate counts gave an estimate of 61,600,000 organisms per cc. in the original suspension. 1 cc. of 1-4,000 dilution in mucin was arbitrarily selected as a test dose and was administered intraperitoneally 2 hours after administration of the serum dilutions, which were given intraperitoneally in 0.5 cc. amounts. The results, given in Table IX, show definite protection in the mice receiving anti-*H. influenzae* horse serum, compared to those receiving normal horse serum.

The above results thus indicate that it is possible to protect mice passively against a lethal infection with *H. influenzae*. Methods of titrating more accurately the protective action of antisera are being investigated further and will be reported in a subsequent publication, together with the results of active immunization experiments.

DISCUSSION

The above results demonstrate that by the aid of mucin a fatal infection with smooth strains of *H. influenzae* can be induced in mice. Such infection is characterized by rapid invasion of the blood stream resulting in a bacteremia, and *in vivo* multiplication of the organism as demonstrated in the present instance by 11 successive mouse to mouse passages. The occurrence of a true septicemia, with multiplication of the bacilli in the blood stream, seems probable but has not been proved, since it is possible that the organism multiplies in the peritoneal cavity with secondary invasion of the blood stream.

The work of other investigators establishing the value of a homogeneous, inbred stock of mice for experiments of this kind has been confirmed. With such a stock it is possible to obtain more consistent results by reducing to a minimum, although not eliminating, spotted deaths due to individual variations in the host.

By means of this method of determining virulence, it is possible to demonstrate considerable differences in the capacity of various meningeal strains of *H. influenzae* to kill mice. Furthermore, the mucin virulence of such strains can be increased by repeated mouse passage until apparent fixation occurs. The strains examined approach this

point to a greater or less degree, some being very nearly as virulent as the fixed strain; others, considerably less so. These same strains without mucin, however, kill mice only in enormous doses, and show no significant difference in their ability in this respect—an added indication that such lethal effect is due chiefly to the inherent toxicity of the organism.

The mechanism by which mucin augments the virulence of smooth strains of *H. influenzae* is as yet undetermined. The fact, however, that this organism is presumably destroyed by complemental bacteriolysis, without phagocytosis, does not support the theory of Nungester, Jourdonais, and Wolf that mucin interferes with the bactericidal properties of phagocytic cells in destroying bacteria. The mechanism of disposal of this organism, as shown *in vitro* by Ward and Wright (11), apparently is not phagocytic but bactericidal.

Furthermore, these experiments indicate that by this method it is possible to demonstrate passive protection of mice infected with mucin suspensions of *H. influenzae*. In addition, this protective action of appropriate antisera can be titrated. Thus this method promises to be of value in assaying the potency of immune sera for clinical use. This method may also be utilized to investigate active immunization in animals.

The results of further studies made possible by the use of the mucin technique will form the subject matter of later communications in this series, *i.e.*, (a) the comparison of the virulence of smooth and rough strains; (b) the mechanism of the action of mucin; (c) a comparison of the susceptibility of inbred mouse strains to this organism; (d) the value of passive immunization in titrating the protective activity of immune sera; and (e) the problem of active immunization.

SUMMARY

1. It has been shown that the ability of virulent *H. influenzae* to kill mice is enhanced by suspending the organism in mucin.
2. The occurrence of a true infection characterized by *in vivo* multiplication of the organism has been established.
3. The necessity for using a pure inbred strain of mice for this type of study has been confirmed.

4. The increase in mucin virulence of a strain of *H. influenzae* by repeated passage through mice has been shown.

5. The usefulness of this method in passive immunization studies has been demonstrated.

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THE PROBABLE NATURE OF THE INFECTIOUS AGENT OF TRACHOMA*

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The confusion and contradiction attending upon the etiology of trachoma when this investigation was first undertaken in 1931 dictated the desirability of establishing certain fundamental conditions regarding the nature of the disease. Consequently, it was necessary in the earlier studies to repeat a number of experiments already described in the literature¹ and to extend them when warranted. In addition, other experiments have been done for the first time in this laboratory, and as a result, the causative agent of trachoma appears to be more tangible and more closely definable.

In the work conducted thus far in this laboratory, it has been demonstrated, as others showed before us (1), that material derived from the conjunctiva of patients with trachoma, while completely innocuous for dogs, rabbits, hogs, guinea pigs, rats, and mice, induces on the conjunctiva of monkeys an infection characterized essentially by folliculosis (2). Preceded by an incubation period of several days to a month, the experimental disease frequently extends from the inoculated to the uninoculated eye and endures for a few weeks to many months, in some instances 2 and 3 years. The histological changes, although simulating those in trachoma, are not emphasized, because follicular reactions of the conjunctiva in general lack any features distinguishing the one from the other. Inoculation of

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¹ It would be impossible in a report of this kind to attempt more than a cursory reference to the extensive literature bearing on the various aspects of the etiology of trachoma. A thorough review of the literature, with a more or less complete bibliography, will be found in a forthcoming monograph on the etiology of trachoma by one of us, to be published by the Commonwealth Fund Division of Publications of New York.

trachomatous material in tissues other than the conjunctiva was found to be without effect. Tissues from different patients vary greatly in infective capacity, and despite the caution exercised in selecting only recent and clinically active disease, about half the tissues only are infectious. Conversely, individual animals (*Macacus rhesus*) exhibit a marked difference in susceptibility, approximately half the monkeys being resistant. It has not been possible to adapt the infection permanently in monkeys, since usually by the third serial passage the infectious agent is either lost or completely inactivated. Upon recovery, no resistance to infection or immunity to the experimental disease is demonstrable. Up to the present time, a hundred or more infected animals have been examined for the epithelial cell inclusions frequently observed in human beings, but neither scrape smears nor histological sections have revealed their presence.

Since the experimental disease lacks the two distinguishing signs of spontaneous trachoma (*i.e.*, cicatrization and pannus), experiments were performed (3) to indicate by virtue of its specificity the genuineness of the disease. Thus, human folliculosis, which in appearance may resemble the experimental disease, is not transmissible to monkeys; the formation of follicles is not stimulated by non-specific infection with a variety of bacteria, by irritation, or by autoinoculation of follicles occurring spontaneously in certain monkeys.

Experiments devised to elucidate the effect of faulty diet on the experimental disease (4) consisted of inoculating monkeys maintained on diets deficient in vitamin A, or inadequate qualitatively and quantitatively in protein, or inadequate in protein and high in fat, or high in fat. The data indicate clearly that a state of malnutrition, as described with its resultant debilitation, does not predispose monkeys to trachoma; on the contrary, the animals appear to be less reactive than those maintained on an adequate diet.

Subsequently, in attempting to define the infectious agent of trachoma, an elaborate study was made of the bacteria cultivable from the disease. A number of workers have ascribed the causation of trachoma to different specific organisms (5), while other authors failed to find any characteristic bacteria (6). It was found in this laboratory (7) that trachoma is not characterized by any particular flora, since the same bacteria may be isolated in similar frequency from other conditions of the eye, and to a less extent even from normal eyes. The variety of bacteria is extensive, and it remains unchanged in the different stages of the uncomplicated disease and is unrelated to the presence of inclusions. Inoculation of the bacteria cultivated, either individually, pooled, or in conjunction with Berkefeld filtrates of infectious material, is without effect on monkeys, even though the tissues from which they are derived are of demonstrable specific infectivity.

The question of whether the infectious agent of trachoma is filterable has occupied the attention of previous workers. Some have succeeded (8) in transmitting trachoma with bacteriologically sterile filtrates, while others found such filtrates to be inactive (9). In any case, however, the number of experiments conducted by the majority of the workers has been too small to include the different variables present in tissue infectivity and animal resistance. Filterability of

the infectious agent was also studied in this laboratory by filtration through Berkefeld V filters. Of 14 experiments performed (10), the original material was infectious in 11. Filtrates of these tissues failed to infect monkeys of demonstrated susceptibility in 10 experiments, and in one the filtrate was as actively infectious as the material from which it was obtained.

The evidence suggests, therefore, that the infectious agent of trachoma is not dependent upon a previously existing state of malnutrition for implantation on the conjunctiva, that it is not bacterial in nature, and that under special conditions it is capable of traversing kieselguhr filters. Because of the obvious indications, a concentrated effort has been made to determine whether the incitant of trachoma may be considered a virus. The experiments undertaken in this connection are presented in the present report.

EXPERIMENTAL

The methods employed both in obtaining material from patients and in inoculating animals² have been described in detail in previous communications, so that a brief recapitulation will be sufficient at the present time. The tissue, removed under novocaine anesthesia from the conjunctiva of each patient by grattage, was suspended in 1.5 cc. of veal infusion broth (pH 7.8). Suspensions from different patients were frequently pooled and triturated under sterile conditions and the ground material was inoculated conjunctivally in monkeys. The inoculations consisted of application of trachomatous tissue by swabbing the everted conjunctiva, or subconjunctival injection alone; or first effecting multiple pricking of the conjunctiva with the charged needle, and then injecting subconjunctivally. No particular differences were noticed in infectivity referable to method of inoculation.

Purification of the Infectious Agent by Testicular Passage

The expedient employed by Noguchi (11) in adapting vaccine virus to rabbit testicular tissue offered the possibility of purifying the infectious agent of trachoma by similar means. For this purpose, material from patients was inoculated intratesticularly in rabbits (12), monkeys, and guinea pigs. Since, however, the most consistent results were obtained in rabbits, the routine of inoculating these

² The human material employed in this study was obtained from the Trachoma Hospital at Rolla, Missouri, and Richmond, Kentucky, and we express our indebtedness with gratitude to Drs. C. E. Rice and J. E. Smith, and Dr. Robert Sory, respectively.

animals alone was employed. To illustrate the effectiveness of this method, a typical experiment will be described in detail.

Material obtained by grattage from two patients was collected in infusion broth as described above. The suspensions were pooled, and after 4 hours required for transportation to the laboratory, two rabbits were injected into the left testicle with 0.5 cc. each, and several monkeys were inoculated by swabbing the upper conjunctiva of both eyes. Within 2 weeks, typical follicles began to appear at the internal angles of the upper conjunctiva, and within a month there was a scattering of follicles over the conjunctiva of all four lids. The infection subsided within 2 months. 1 week following inoculation, the inoculated testicle of one rabbit was removed, with precautions for sterility, and subsequently ground in a sterile mortar. The emulsified tissue was then inoculated in monkeys by swabbing and by injection. 2 weeks following inoculation, the second rabbit was studied in the same way, and inoculations were repeated in monkeys. The results of the rabbit passage showed that the testicular material 1 week following inoculation was moderately infectious and the disease persisted from 1 to 2 months; the material 2 weeks after inoculation was most virulent of all, the incubation period being shortened to 5 to 7 days and the infection persisting for several months. This increased virulence is only apparent, however, since other materials showed differences in one direction or the other which were undoubtedly referable to variation in individual susceptibility of monkeys.

Four typical experiments are summarized in Table I. An analysis of the data reveals that in 2 experiments the human material was originally infectious and retained its infectivity after testicular passage in one but not the other instance. The material retaining its activity showed no inclusions, while that losing its infectivity contained typical inclusions. In 2 other experiments the human material did not infect monkeys, but in one the infectious agent was demonstrable following testicular passage. The material exhibiting infectious properties following passage contained no inclusion bodies, while the other did. In no case, however, were inclusion-like structures seen in preparations from either rabbits or monkeys.

Twenty experiments in all were performed in similar fashion. The material came from single cases, or that from 2 to 9 patients was pooled. In 10 experiments, the original material proved non-infectious, while in the remaining 10, follicular reactions, characteristic of experimental trachoma, were induced in monkeys following their inoculation. Of the 10 materials originally non-infectious, 2 were capable of infecting monkeys specifically after testicular passage, and

8 remained non-infectious. With the 10 materials of demonstrated infectivity, 4 were unable to infect monkeys after testicular passage, and 6 retained their ability to infect. In attempting to transmit the infectious agent in series, it was found that by the third intratesticular passage it was lost or completely attenuated. The discrepancies measurable in terms of infectivity preceding and following testicular passage serve to demonstrate in another way the difference in resistance of individual monkeys, rather than an acquisition or loss of infectivity by the original tissues. In fact, it is because of this marked variation in susceptibility, as already pointed out, that in order to avoid inaccurate conclusions, experiments on trachoma should be carried out on a statistical basis.

TABLE I

Purification of the Infectious Agent of Trachoma by Rabbit Testicular Passage

Experiment No.	No. of cases pooled	Presence of inclusions	Infectivity		Presence of inclusions in	
			Before passage	After passage	Rabbit	Monkey
T36	2	Both negative	Positive	Positive	Negative	Negative
T58	2	Both positive	"	Negative	"	"
T49	2	Both positive	Negative	"	"	"
T53	3	All negative	"	Positive	"	"

In addition to the experiments with material from human beings, a study was also made on the intratesticular passage of infected tissues from monkeys (*Macacus rhesus*). Material was obtained in the same way, and 4 experiments were done in all. In each instance, the presence of the infectious agent after passage was demonstrated by subsequent infection of monkeys.

For purposes of control, material obtained in the same way from ten patients with folliculosis and from two patients with a chronic infectious conjunctivitis of undetermined etiology was studied in a similar manner. But on no occasion was it possible to demonstrate the presence of an infectious agent in rabbit testicle. Since it has been reported (13) that epithelial cell inclusion bodies indistinguishable from those encountered in trachoma may be found in scrape smears of the

conjunctiva during hog cholera, another experiment was performed with hog cholera virus, which was first proved fatal for hogs. When inoculated directly or following passage in rabbit testicle, it failed to invoke any significant reaction in the conjunctiva of monkeys.

Before preparing the testicular tissue for inoculation, sufficient tissue was removed for histological and bacteriological study. Tissue cultures were also made, as will be described later. Impression smears from cut surfaces were stained by Gram for bacteria and by Giemsa for inclusion bodies. Except for a minor degree of inflammatory reaction in occasional rabbits, probably referable to adventitious bacteria present in the original tissues, the tissue changes were insignificant. This is in agreement with the clinical observation that none of the rabbits injected with trachomatous tissue gave any evidence of a general or local reaction. Inoculations made on several different bacteriological media yielded no growth, except on rare occasion when a colony or two of *Staphylococcus albus* was isolated. Tissue cultures made at the same time were also free of bacteria following incubation. Bacteria were never demonstrated in the impression smears, and neither the elementary nor the initial bodies associated with the inclusion body of trachoma were ever found.

It is interesting in this connection that in attempting to preserve the infectious agent of trachoma under a variety of conditions, Nicolle and Cuenod (14) found that in a single experiment trachomatous material retained its activity 37 days after inoculation into rabbit testicle. Subsequently, Nicolle reported (15) that infectivity was not demonstrable 30 days after testicular inoculation. In each instance, details are lacking as to infectivity of the trachomatous tissues preceding inoculation of the rabbit, presence of bacteria before or after passage, clinical effect in rabbits, and histology of the inoculated testicle.

The purification and survival of the infectious agent in rabbit testicle suggested the possibility of adapting it to brains of rabbits. Material from patients was therefore injected intracerebrally, and after an interval of 2 weeks the animal was sacrificed and the brain employed for serial passage, inoculation of monkeys' eyes, and further study. In 5 experiments of this kind, it was found that the infectious agent survived once, although inducing no tissue alteration. Further

attempts at cerebral adaptation indicated that, although occasionally encephalitic lesions due to *Enccephalitozoon cuniculi* (16) were encountered, the infectious agent became lost in serial passage. Similar efforts to adapt the infectious agent to brains of monkeys were also failures.

So, also, passing infectious testicular tissue intraperitoneally through mice rendered the infectious agent recoverable only rarely within the first 24 hours by washing out the peritoneal exudate. The mice suffered no noticeable inconvenience, and impression smears of several organs revealed nothing of note.

Consequently, the evidence indicates, as observed in the one positive filtration experiment cited above, that it is possible to infect monkeys with experimental trachoma with material completely liberated from extraneous bacteria as determined by smear, bacteriological and tissue cultivations. The absence of any reaction in the rabbit, the inability of the infectious agent to survive serial passage, and the maintenance of infectivity in many instances at a given level, all suggest that the infectious agent of trachoma does not propagate in the testicle but is merely preserved. During the period of intratesticular passage, however, purification from the cultivable bacteria present in trachomatous eyes is achieved. Therefore, while this method of demonstrating the presence of the agent of trachoma is irregular in occurrence, it nevertheless justifies its original purpose of excluding bacteria a sufficient number of times (12) to indicate that the organisms cultivable from the trachomatous conjunctiva are not responsible for the infection.

Additional Studies on Filterability

With the tentative elimination of bacteria as involved in the evolution of trachoma, it became desirable to restudy the question of filterability of the incitant of the disease. In retrospect it seemed that several possibilities, all amenable to experimentation, might explain the frequent failure of successful filtration. Adsorption on the filter, reverse potential of the infectious agent, the presence of the infectious agent within or closely adherent to the epithelial cell, etc., theoretically, at least, might interfere with the filtration of an otherwise filterable agent. A publication in the meantime, by Thygeson and his associates, suggested that non-filterability was in fact due to

the adsorptive properties of kieselguhr filters. While in a previous study (17) with Berkefeld and Chamberland filters he had reported that the infectious agent of trachoma was not filterable, he demonstrated at this time (18, 19) that collodion filters as devised by Elford (20) allowed the agent to pass. Both Cattaneo (21) and Stewart (22), on the contrary, failed to show that filtration was any more successful with collodion membranes.

Subjecting the question of filterability to restudy, then, it was decided to consider the various possibilities suggested and to conduct filtrations, when material was sufficient, under different conditions. Accordingly, four different filters were employed: Berkefeld V, Seitz, plaster of Paris as fashioned by Kramer (23), and collodion membranes.³

In the majority of instances, the material was collected in infusion broth, but on occasion in Tyrode solution. Scrapings from several patients were pooled and ground in a sterile mortar 3 to 4 hours after collection. After grinding, the suspension was centrifugated for 5 minutes at about 1500 R.P.M. This was sufficient to sediment the various tissue cells and yet not destroy the infective capacity of the supernatant fluid, which was subsequently used for filtration. In each case, filtration was accomplished in a few minutes under a pressure of about 20 cm. of Hg. Cultures were made of the filtrates, and the integrity of the filters was tested later with broth cultures of *Bacillus prodigiosus*; the plaster of Paris filters were controlled by passage of Congo red. Inoculations were then made in monkeys with unfiltered material, with supernatant after centrifugation, and with filtrate.

Twenty-two experiments were done in all, but since the unfiltered material was infectious in only 9, obviously the other experiments have little significance. In all 9, filtration was done with collodion membrane of an average pore size close to 0.6μ in the different tests. In all cases the filtrates failed to infect monkeys. In 5 experiments, parallel filtrations were carried out with Berkefeld V and collodion filters, and in 4, filtrations were done simultaneously with all four varieties of filter. To illustrate experiments conducted on parallel filtration, a summary protocol is given in Table II. It will be seen on examination that in the 4 experiments material was pooled from 4 to 9 patients, with inclusion bodies varying in a percentage incidence of 20 to 75.

³ The filtering apparatus used was a replica of a model kindly supplied for reproduction by Dr. J. H. Bauer of The Rockefeller Foundation (24).

In each instance, the unfiltered material and the supernatant following centrifugation were infectious in monkeys, thus showing that grinding and centrifugation did not alter measurably the original infectivity. In each case, however, the filtrates from Berkefeld V, Kramer, Seitz, and Elford filters were incapable of inducing experimental trachoma. A number of the monkeys which proved to be non-reactive to filtrates were infected at a later date with unfiltered material, thus eliminating the possibility that their natural resistance was responsible for the original failure of infection. Since the study of filtration is still in

TABLE II
Filterability of Infectious Agent of Trachoma

Experiment No.	No. of cases pooled	Presence of inclusions	Infectivity of					
			Unfiltered material	Super-natant	Berkefeld filtrate	Kramer filtrate	Seitz filtrate	Elford filtrate
T64	9	Present in 6 of 9 patients	Positive	Positive	Negative	Negative	Negative	Negative
T65	4	Present in 3 of 4 patients	"	"	"	"	"	"
T66	5	Present in 1 of 5 patients	"	"	"	"	"	"
T67	5	Present in 2 of 5 patients	"	"	"	"	"	"

progress, it is not desirable to draw final conclusions, although the indications are that filterability appears to be as difficult of achievement as was originally stated.

In addition to the experiments with human tissues, a number of attempts were made to filter infected animal tissues. Scrapings from the conjunctiva of monkeys (*Macacus rhesus*), as well as ground rabbit testicle, were filtered similarly to the human material through both Berkefeld V and Elford filters. The results were uniform in indicating a complete loss of infectivity by the filtrates of tissues from monkeys and rabbits.

There are several reasons for assuming that trachoma is preeminently an infection of the epithelial cell. If this assumption is correct, it should follow logically that the infectious agent is contained within

the cell or attached to its surface. Since the methods employed for filtration retain all tissue cells, it is possible that in consequence the virus, if not entirely withheld, may be diminished to a point beyond its range of infectivity. In order to test this hypothesis, several experiments were undertaken in which lysis of the epithelial cell was caused by supposedly gentle means before attempting filtration. Thus, rupture of the cells was obtained with ox bile, dilute alkali, and alternate freezing ($-15^{\circ}\text{C}.$) and thawing ($+8-10^{\circ}\text{C}.$). However, it has been impossible as yet to gain any information on filterability under these conditions, because all the methods thus far attempted have inactivated the infectious agent. Materials tested after the manipulation in question, and before filtration, have always failed to infect monkeys of proved susceptibility.

Cultivability of the Infectious Agent

The results already described in connection with testicular passage reveal the inability of the infectious agent of trachoma to multiply in the usual bacteriological media. It was proposed, therefore, to study cultivability in tissue cultures. For this purpose, the technique devised by Maitland and Maitland of minced rabbit kidney (25), and as modified by Li and Rivers (26) of minced chick embryo, the developing chick egg (Woodruff and Goodpasture (27); also Burnet and Galloway (28)), and various tissue fragments in plasma, were particularly utilized. In order to control both the methods and conditions of growth, similar experiments were carried out with the virus of St. Louis encephalitis, as already reported (29). These methods of tissue cultivation were supplemented later by cultures made in plasma clots with human placental tissue extract and plasma from patients or normal individuals. In attempting to approximate very closely the conditions found in the eye, epithelial cells containing both the infectious agent (as demonstrated by inoculation of monkeys), and in some cases even inclusion bodies, were seeded by this method.

The technical difficulties accompanying the studies in tissue cultivation were numerous, the most consistent being the presence of adventitious bacteria in the original tissues. In the earlier seedings, they were almost always present in the inocula, and they grew rapidly in culture, with the result that the growing cells were quickly destroyed or suppressed, thus defeating the purpose of the experi-

ment. The use of several methods and chemicals, to inactivate selectively the bacteria only, was of no avail, since the infectious agent proved to be less resistant to the different substances, as will be brought out later. This obstacle was circumvented, however, by careful irrigation of the eye with salt solution, and then making multiple seedings of essentially epithelial scrapings collected in Tyrode solution. While it was not possible to eliminate bacterial growth entirely in this way, bacteriologically sterile tissue cultures were frequently obtained. The seeded cultures were incubated both at 30° or 32°C. (to approximate the temperature of the conjunctiva) and at 35° or 37°C., and incubation was carried out for 3 to 7 days before transplants were made or before the cultures were nourished. Other cultures, under similar conditions, were made from infected eyes of monkeys and from testicular tissue carrying the infectious agent. In testing for infectivity, tissue cultures were inoculated in monkeys before and after transplanting, and in every case the inoculum was derived from several cultures which had been pooled and triturated.

The data bearing on tissue cultivation have been summarized in Table III. Examination of the protocol shows that cultivation was tested in 261 cultures of minced chick embryo representing 43 patients whose conjunctival scrapings were pooled to form 12 experiments, in which the original material was infectious 6 times. In the fertile egg, 91 seedings were made from 26 patients, so pooled as to comprise 10 experiments, in 4 of which the human tissues were of demonstrated infectivity. In minced rabbit testicle, 254 inocula were made from 59 patients, for a total of 18 experiments, in which the original tissues were capable of infecting monkeys 6 times. So, also, in rabbit testicle in plasma clot, 217 separate cultures were grown from material supplied by 68 patients, comprising 20 experiments, in 8 of which the original material induced experimental trachoma. In cultures of minced rabbit kidney, 118 attempts were made to cultivate the infectious agent of trachoma, in 8 different trials, with the human material infectious twice. Cells from the human conjunctiva of 48 patients, pooled to make 13 experiments, were cultivated in clotted plasma derived from the same patient and from normal individuals. In 5 of these experiments, conjunctival cells were derived from patients of proved infectivity. In summary, then, 81 experiments, in 31 of which the original human material was infectious for monkeys, were conducted by cultivating and inoculating tissue from different animal species. From 30 to 40 per cent of the trachomatous materials employed in the different experiments on cultivation were found to

contain epithelial cell inclusions. In none of the cultures, however, was it possible to demonstrate the presence of the infectious agent of trachoma by inoculation of monkeys.

Cultivations attempted on a lesser scale in minced mouse kidney, in guinea pig testicle, in the Brown-Pearce tumor of rabbits, in testicle and conjunctiva of monkeys, were all uniformly negative. In several instances, infectious material was inoculated in rabbit testicle simultaneously with a ground suspension of the rabbit tumor described by Brown and Pearce. Again the results indicate a failure of the infectious agent to grow. At other times, tissue cultivation was carried

TABLE III

Attempts to Cultivate the Infectious Agent of Trachoma in Tissue Culture

Method of tissue culture	No. of patients		No. of experiments with pooled material		Total No. of cultures	Infectivity of tissue cultures
	Studied	With inclusions	Infectious	Non-infectious		
Minced chick embryo.....	43	13	6	6	261	All negative
Fertile egg.....	26	10	4	6	91	" "
Rabbit testicle						
(a) Minced.....	59	20	6	12	254	" "
(b) In plasma clot.....	68	24	8	12	217	" "
Minced rabbit kidney.....	17	7	2	6	118	" "
Human conjunctiva.....	48	19	5	8	234	" "
Totals.....	261	93	31	50	1175	All negative

out anaerobically, as suggested by Dochez, Mills, and Kneeland (30), without, however, affecting the results already observed with other techniques. A few other experiments were attempted with cultivation of human placenta, but these were discontinued because of the difficulties encountered in growing this tissue.

The evidence is clear, then, that despite numerous attempts to cultivate the infectious agent of trachoma under a variety of conditions, in tissues from six different animal species, including man, and a cultivable rabbit tumor, it has not been possible to create *in vitro* the proper conditions for multiplication. In a recent preliminary communication from India, successful cultivation of the trachomatous agent

in the fertile egg has been reported (31). The evidence presented, however, is not convincing, since the measure of propagation was not infectivity in man or monkey, but the appearance of gross lesions on the chorioallantoic membrane. There was no information in the report to indicate whether even these changes might not have been due to bacterial contamination.

Characteristics of the Infectious Agent of Trachoma

The studies presented up to this point have dealt mainly with the grosser characters of the infectious agent of trachoma, particularly its ability to induce specific infection in monkeys. While at the same time they reflect the nature of the agent, they do not furnish sufficient detailed information to make clear its character. Consequently, experiments were performed on its inactivation or ability to survive under different circumstances. Recognizing the danger of generalization, since the variation both in infectivity of tissues and in susceptibility of animals is great, it nevertheless seems fair to depict the characteristics to be described as typical of the infectious agent of trachoma.

Inactivation by Heat.—As in all the experiments to follow, the reactions were studied with material obtained from patients by grattage and suspended in infusion broth. Consequently, the infectious agent was present in a mixture of lachrymal secretion, blood and tissue cells, consisting chiefly of epithelial cells and to a much less extent of lymphocytes and monocytes. How much of a protective influence the different constituents afforded the infectious agent is obviously indeterminable under the present conditions of experimentation. Such suspensions, then, were placed in a water bath regulated at different temperatures (40°, 45°, 50°, 55°, 60°C.) and for varying intervals of time (15, 30, 45 minutes). In later experiments, the two higher temperatures and the 45 minute interval were discontinued. At the end of the exposure, the material, after cooling, was inoculated in monkeys simultaneously with the unexposed material in control animals. The experiment was repeated a sufficient number of times to allow the conclusion that exposure at 45–50°C. for 15 minutes regularly inactivates the infectious agent. So, also, Hess and Römer (32), and Botteri (33), found that $\frac{1}{2}$ hour at 50°C. destroys the infectious agent.

Preservation.—Preservation of the infectious agent of trachoma in glycerine (34) has been reported by several workers, with a remarkable difference of opinion (35). In most cases, however, the data are difficult of analysis, since little effort was made to determine either the original infectivity of the material or preservation under the same conditions without glycerine. In these experiments, the infectivity of the original suspensions was tested, and when they were found to be non-infectious, the data were discarded. Since variations were encountered in different brands of glycerine, that prepared by Schering-Kahlbaum of Berlin was used, as best adapted for this work. The results of repeated experiments indicate that at ice box temperature glycerine does not maintain the infectious agent active any longer than preservation without glycerine. Ordinary preservation at this temperature varies, with different suspensions, from 1 or 2 days to a week or more. In general, however, tissues lose their activity within 3 or 4 days under these conditions. Similar variations in maintenance of infectivity were observed at room temperature, the infectious agent remaining active for a few hours, varying from 2 or 3 to rarely 24 hours. At incubator temperature ($37^{\circ}\text{C}.$), inactivation occurs within a few hours.

Effect of Chemical Agents.—Cell suspensions containing the infectious agent were tested for its ability to survive some of the commoner chemical agents. Ox bile (36), added to the amount of $1/4$ to $1/3$ the volume of the suspension, was found to inactivate the infectious agent after an exposure of 15 minutes at $37^{\circ}\text{C}.$ At the end of this interval, practically all the cells were lyzed, and the inclusion bodies, when present, were dissolved, thus disappearing, while, as might be expected, many of the bacteria originally present were still cultivable.

Silver nitrate, which is commonly used in the treatment of trachoma, was also tested for its effect on the infectious agent. In a final concentration of 2 per cent, this reagent causes a heavy coagulation of the suspensions, and the infectious agent is inactivated regularly within 3 to 4 hours.⁴ So, also, cocaine was found to be deleterious, so that

⁴ The regularity of time exposure in these experiments is accounted for by the fact that after collecting the material at the Rolla hospital, it had to be transported to St. Louis before inoculation in monkeys was possible. This was done in most of the experiments recorded in this report, and the material was kept on ice during transportation.

this anesthetic was never employed when obtaining material from patients. In concentration of 4 per cent, cocaine inactivated regularly, while in 2 per cent concentration inactivation was effected after an interval of 3 to 4 hours in about one-half the tissue specimens. Gentian violet in final dilution of 1:100,000 also destroyed the infectivity of active tissues within the same time interval. Tartar emetic inactivated the agent within this period, when present in an ultimate dilution of 1:1000, as did also a 0.25 per cent solution of phenol.

Immunogenic Properties.—The common clinical history of repeated infection in patients with trachoma was experienced in the experimental work when it was shown that monkeys acquire no active immunity to infection after recovery from the artificially induced disease. Nevertheless, an effort was made to determine whether human sera may act to prevent or diminish experimental infection. Conjunctival scrapings from patients, of verified infectivity, were mixed with sera, whole blood, or plasma, derived in some experiments from the patients themselves, and in others from normal individuals. In most of the experiments, the mixture was kept at 20°C. for periods varying from 4 to 12 hours. In a few experiments, incubation was carried out for $\frac{1}{2}$ hour at 37°C. It was not possible to lengthen the incubation period at body temperature because of the injurious effect of the higher temperatures. The mixtures were then inoculated in monkeys to determine whether they were still capable of causing experimental infection. Not desiring to prolong unnecessarily the presentation of these experiments, it is important to state only that blood from patients with trachoma contains no demonstrable substance capable of inactivating or neutralizing the infectious agent, as measured by the development of experimental trachoma in monkeys.

Relation of the Infectious Agent to the Epithelial Inclusion

The epithelial cell inclusion, first described by Prowazek and Halberstädter, was considered by them as the causative agent of trachoma (37). It will not be possible to review in this report the tremendous literature bearing on this structure. For purposes of orientation, however, it is necessary to point out that the inclusion is composed of heterogeneous elements which may appear relatively large, pleomorphic, and basophilic (*i.e.*, initial bodies), or minute, uniform, coccoid,

and acidophilic (*i.e.*, elementary bodies). Both appear extra- or intracellularly, frequently forming over the nucleus a cap composed entirely of either one form or the other, or even in common agglomeration. As a rule, elementary bodies are less common and are more often extracellular, while the initial bodies are more numerous and more frequently occur intracellularly. The inclusions are found in few numbers in trachoma, and in only roughly half the patients, although their incidence is more frequent in the recent infections.

Attempts made in this laboratory to correlate the presence of inclusions with infectious activity of given tissues indicate that materials containing inclusions are not necessarily infectious for monkeys, and that materials lacking inclusions may be actively infectious. Inclusions have never been found in monkeys successfully infected, in infectious rabbit testicle or brain, in tissue culture, or in various organs of mice injected intraperitoneally.

The literature indicates a division of opinion among different workers, the earlier apparently finding inclusions in infected animals (38), while the later have been unsuccessful (39). The inclusions found by the earlier investigators were found, in general, shortly after inoculation with whole material, even in the absence of the experimental disease (Halberstädter and von Prowazek, Leber and von Prowazek, Herford, etc.), and their presence was transient. In successful transmission in monkeys with filtered material, inclusions either were not found (Bertarelli and Cecchetto, Julianelle and Harrison) or they were not sought for (Nicolle, Cuenod, and Blaisot; Olitsky, Knutti, and Tyler; and Thygeson and Proctor). In this connection, however, it is important to recall the study of Thygeson, Proctor, and Richards (19), in which they showed that a bacteriologically sterile filtrate of human trachomatous tissue obtained by filtration through a collodion membrane of 0.6μ A.P.D. not only induced trachoma in a human volunteer, but stimulated the formation of epithelial cell inclusions from the 5th day on. The filtrate contained minute structures which the authors considered to be elementary bodies.

The evidence to be gained from the literature indicates that in all the successful transmissions practiced in man, with unfiltered material, to be sure, inclusions were always found when sought. The information available at the present time, however, does not allow a categorical statement regarding the nature of the inclusion body. Whether it represents the infectious agent itself, as the experiment of Thygeson, Proctor, and Richards suggests, or a reaction product between cell and

agent; or whether, as some believe by inference rather than experimentation, it is another agent concurrently present, or consists of nests of phagocytized bacteria, must await future work for elucidation.

DISCUSSION

As a result of continuing the preceding studies on the infectivity of tissues from patients with trachoma, it has been possible to visualize more concretely the infectious agent of this disease. Adapting to this problem the technique of testicular passage in rabbits, it was found that trachomatous tissues, from both man and monkey, may be purified of the extraneous bacteria usually present on the conjunctiva. While irregular in its execution, this method permits the infectious agent to retain its infectivity a sufficient number of times to support the conclusion that trachomatous tissues liberated of bacteria may still be specifically infectious for monkeys. The evidence indicates, however, that the infectious agent does not multiply during this passage.

Further experiments on filtration, employing Seitz, Kramer, Berkefeld, and Elford filters, as described above, justify the impression, gained in earlier studies, that successful filtration is accomplished only rarely and with difficulty.

Except for the studies on filtration reported by Stewart and ourselves, too few experiments have been done by each investigator to warrant the opinion that the infectious agent of trachoma is readily filterable. Thus, Bertarelli and Ccchetto, who first reported successful filtration, did a single experiment; Nicolle, Cuenod, and Blaizot did 2 experiments, both successful; Thygeson and his associates reported 6 tests in human beings, with Berkefeld and Chamberland filters, all negative, 4 experiments with collodion membranes, in baboons, all positive, and later a single test, as cited above, in a human volunteer. Of the others reporting filtration, Olitsky, Knutti, and Tyler demonstrated filterability in 1 of 6 trials, and we, in 1 of 20 trials. This makes no allowance, however, for the imposing number of experiments by ten groups of workers who did not establish filterability. It is suggested, therefore, not that the infectious agent is incapable of filtration, but rather that it is not readily filterable, regardless of the method of filtration employed.

Upon reconsideration, it may be that filtration of the infectious

agent depends upon a certain degree of epithelial cell degeneration for its liberation into the surrounding menstruum. Since cellular degeneration is slight, particularly in the uncomplicated stages, it is not unlikely that the unattached infectious agent occurs in quantities insufficient for successful filtration. In the rarer cases of marked cellular degeneration, on the other hand, the infectious agent is filterable, thus contributing the few examples reported in the literature. In inclusion blennorrhea, a related ocular condition, filtration has been reported with greater regularity. In this infection, excoriation and degeneration of epithelial cells is commonly present, and the inclusion, which is indistinguishable from that in trachoma, occurs not only in greater numbers but frequently extracellularly. Since grinding, with its partial cellular disruption, does not suffice in improving filterability, further experiments were undertaken in this connection, which yielded no information, since the attempts to lyse the cells *in vitro* resulted in inactivation of the infectious agent. Consequently, with experimental evidence lacking, the explanation advanced above remains only speculation.

Inability of the infectious agent to multiply in various bacteriological media suggested attempted propagation in tissue cultures. A number of techniques were employed for this purpose, with both infectious and non-infectious human material, which in 35 per cent of the cases contained inclusions, as reported in preceding pages. None of the methods, however, supplied the proper conditions for growth. The conclusion is inevitable, therefore, that the infectious agent of trachoma possesses an exquisite tissue specialization. Unable to infect lower animals at all, the modified disease it induces in apes and monkeys is confined to the conjunctiva. In man, the infectious agent finds the best conditions for growth, and yet the disease is of protracted onset and an extremely circumscribed localization. It is not surprising that it fails to grow in cultures of avian or mammalian tissue. It was for this reason that the technique was conceived of cultivating, in homologous plasma, epithelial cells from the trachomatous conjunctiva, which were frequently infectious and even contained inclusion bodies. While this appears to be a close *in vitro* approximation of the agent's normal habitat, propagation did not ensue.

Upon careful reflection, the evidence suggests that the infectious agent of trachoma is a virus, filterable with difficulty, under conditions

not yet understood. Its characteristics of low infectivity, marked tissue specialization, poor immunogenic properties, rare filterability, weak propagative power, fragility before different agents, all classify the virus as an extremely unusual variety. Indeed, even the inclusions accompanying its presence in human tissues differ from the virus inclusions heretofore recognized. If future investigation succeeds in confirming the viral nature of the infectious agent of trachoma, it will have to be regarded as possessing properties differing considerably from those of viruses now generally known.

SUMMARY AND CONCLUSIONS

1. The infectious agent of trachoma can be freed from extraneous bacteria by passage through rabbit testicle.
2. The infectious agent multiplies little, if at all, during such passage, but in many instances retains its infectivity undiminished.
3. No specific changes occur in the rabbit testicle incidentally to the passage.
4. On rare occasion the trachoma agent may be freed from bacteria by intracerebral passage. The brain tissues show no specific reaction.
5. Filtration experiments with Seitz, Kramer, Berkefeld, and Elford filters confirm the general observation that the infectious agent is filterable with difficulty.
6. Tissue culture experiments, with tissues containing the infectious agent (conjunctiva, rabbit testicle, brain, etc.), conducted under a wide variety of conditions, proved uniformly unsuccessful in the cultivation of the agent.
7. The agent is inactivated by bile, AgNO_3 , phenol, cocaine, tartar emetic, and gentian violet. Its heat inactivation temperature is between 45° and 50°C. , at a time interval of 15 minutes.
8. Attempts to preserve the infectious agent in glycerine were unsuccessful.
9. The accumulated evidence suggests that the infectious agent of trachoma is a virus.

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STUDIES ON THE SUPRARENAL CORTEX

VI. THE EFFECT OF SUPRARENAL CORTICAL HORMONE UPON THE ELECTROLYTE EXCRETION OF THE INTACT NORMAL DOG.

A PROPOSED METHOD OF COMPARATIVE ASSAY

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CORRECTION

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Page 501, Table I, 3rd column, 3rd line of Experiment II, for 12/19/21 read
12/19/13.

Healthy, male dogs weighing approximately 10 to 15 kilos were used throughout this study. Routine care of the dogs and conduct of the metabolism studies have been described elsewhere (2). The daily quantity of water and raw beef necessary to maintain constant body weight was determined during a preliminary

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¹ We acknowledge the assistance of Dr. George Cartland of the Upjohn Company, Kalamazoo, Michigan, Dr. Oliver Kamm of Parke, Davis and Company, Detroit, Michigan, and Dr. David Klein of the Wilson Laboratories of Chicago, who have supplied us with generous amounts of suprarenal cortical hormone.

period. In addition to water and meat, the dogs received an arbitrary amount of sodium chloride (2.5 or 3.0 gm.) as a 10 per cent solution, 50 cc. of a 10 per cent solution of dried milk powder, and a capsule of haliver oil.²

The dogs were catheterized each morning and the bladder was washed three times with 10 cc. of distilled water. The washings were added to the 24 hour urine specimen. Catheterization of male dogs was accomplished easily by using a No. 7 French silk catheter. A size 18 needle was introduced into the catheter. This furnished an excellent adapter for a 20 or 50 cc. syringe. The care of the catheters was considered particularly important. They were kept in a solution of bichloride of mercury and rinsed carefully with distilled water before being used. At the termination of each experiment (usually a 3 day period) 20 cc. of a 10 per cent solution of argyrol were introduced into the bladder of the dog. The same dogs were catheterized daily for a period of several months without developing bladder or renal infection. The urinary sediment was examined frequently.

Following catheterization the extract was given. When divided doses were administered, one-half of the total amount of extract was given immediately. The remainder was given approximately 7 hours later. On the control days an equivalent amount of sterile normal saline solution was administered to compensate for the sodium chloride contained in the extract. 3 days were found to be an ample interval for the return to normal state.

Within $\frac{1}{2}$ hour after the extract was given, a stomach tube was used to give the dogs the daily ration of milk, sodium chloride solution, and water. Care was taken to introduce first the milk, then the sodium chloride solution, and finally the water. The milk and sodium chloride solution were measured with volumetric pipettes. The water was used to rinse the stomach tube. Immediately following this procedure, the dogs were fed the ground beef and capsule of haliver oil. Aliquot portions of the same batch of raw beef were supplied during the period of study.

During the 3 hour experiments the dogs rested quietly on a table. Indwelling catheters were used.

Methods of Analysis

The urine specimens were preserved with toluol. The methods used for the blood and urine analysis of sodium, potassium, chloride, total nitrogen, inorganic phosphate, creatine, and creatinine have been described previously (2, 4). Potassium determinations were made on specimens ashed with thorium nitrate according to the method of Strauss (5). Calcium was analyzed according to a modification of the micro method of Tisdall and Kramer (6). Magnesium was determined according to the method of Briggs (7).

² Haliver oil capsules were supplied by Dr. Oliver Kamm of Parke, Davis and Company.

EXPERIMENTAL

Sodium excretion was decreased during the first 3 hour period (Table I) following the subcutaneous injection of 2000 dog units of suprarenal cortical hormone.³ This reduction was more pronounced between the 4th and 6th hours. The effect of the hormone was reflected in the 24 hour excretion of sodium. The decreased sodium excretion represented an actual reduction in concentration, notwithstanding lowered urinary volume. In contrast to sodium, the potassium excretion was increased. Injections of hormone were accompanied by no change, an increase, or a decrease in urine volume (Table II). The action of the hormone on the renal excretion of sodium and potassium was independent of alteration in urine volume.

TABLE I

The Effect of a Single Subcutaneous Injection of Suprarenal Cortical Hormone on Renal Excretion of Sodium and Potassium in the Normal Dog*

Hour	Urine volume		Sodium		Potassium	
	Control	Treated	Control	Treated	Control	Treated
	cc.	cc.	m. eq.	m. eq.	m. eq.	m. eq.
9 a.m.-12 m.	18	24	4.8	2.8	0.9	2.1
12 m.-3 p.m.	18	55	3.9	0.3	0.6	0.5
3 p.m.-9 a.m.	320	180	56.2	27.9	15.4	20.3
24 hr. total.....	356	259	64.9	31.0	16.9	22.9

* 2000 dog units of suprarenal cortical hormone were injected at 9 a.m.

When a single, daily subcutaneous injection of hormone was employed, large quantities were required to produce a definite change in the excretion of electrolytes during the 24 hour period. Much smaller quantities of extract were effective when administered twice daily. The fact that multiple injections of the hormone were more effective than an equivalent single dose confirms the clinical observations in the treatment of Addison's disease.

Suprarenal cortical hormone (300 to 500 dog units injected subcutaneously twice daily) produced a significant decrease in the excretion

³ These dog units refer to the method of standardization on suprarenalectomized dogs employed by the manufacturers of the extract.

of sodium and chloride in four normal dogs (Table II). Potassium excretion was increased and inorganic phosphate excretion was usually increased. Total nitrogen excretion was increased in one dog (1-75) but not significantly altered in the others. Calcium and magnesium excretions were increased during the day in which extract was injected.

TABLE II

The Effect of Divided Subcutaneous Injections of Suprarenal Cortical Hormone on Renal Excretion of Electrolytes in the Normal Dog*

Dog	24 hr. period	Urine volume	Sodium	Chloride	Potassium	Calcium	Magnesium	Inorganic phosphorus	Nitrogen	Dosage of extract
		cc.	m. eq.	m. eq.	m. eq.	m. eq.	m. eq.	mg.	gm.	
1-75	Control	548	64.8	70.3	22.6	1.4	0.8	626	11.5	Normal saline
	Treated	556	55.3	51.4	35.2	2.0	1.1	722	13.2	1000 dog units
	Control	544	71.0	71.2	28.8	1.3	0.9	538	11.4	Normal saline
1-78	Control	630	78.8	78.2	27.3	0.4	1.1	642	12.1	Normal saline
	Treated	604	52.6	64.6	42.4	0.6	1.1	744	12.5	600 dog units
	Control	570	88.6	76.8	18.2	0.1	0.9	570	12.2	Normal saline
1-79	Control	1230	58.8	59.6	21.7	0.2	1.2	497	10.9	Normal saline
	Treated	1110	38.6	42.6	33.1			522	10.0	800 dog units
	Control	1210	76.0	69.4	26.5	0.3	0.8	410	9.2	Normal saline
1-80	Control	393	62.2	64.8	20.5		0.6		10.7	Normal saline
	Treated	530	55.2	57.4	24.5		0.8		10.8	1000 dog units
	Control	472	71.8	71.0	13.5		0.7		10.5	Normal saline
Total	Control	2801	264.6	272.9	92.1	2.0	3.7	1765	45.2	
	Treated	2800	201.7	216.0	135.2			1886	46.5	
	Control	2796	307.4	288.4	87.0	1.7	3.3	1518	43.3	

* The extract was injected in divided doses twice daily.

Frequently an amount of sodium, quantitatively equal to that retained, appeared in the urine on the day following hormone administration (Table II). During this same period potassium excretion was reduced. The alteration in potassium excretion was not so constant as that in sodium. This "rebound" effect proved a useful check on the alterations produced by the cortical hormone. In an isolated

TABLE III
*The Effect of Continued Subcutaneous Injections (5 Days) of Suprarenal Cortical Hormone on Renal Excretion and Blood Constituents in the Normal Dog**

Period	Duration	Body weight	Urine volume	Sodium per day	Chloride per day	Inorganic phosphate per day	Nitrogen per day	Creatinine per day	Creatinine per day	Red blood cells	Hemoglobin	Volume packed red blood cells	Plasma proteins	Sodium per liter plasma	Chloride per liter plasma	Blood sugar	Blood urea	Treatment
Control	2	13.6	510	83.3	78.7	591.0	13.7	0.444	1.030	7.1	13.8	—	5.4	144.3	114.3	80	20	Normal saline solution
Treated	5	13.4	571	78.1	79.8	682.0	13.3	0.480	0.969	6.3	12.5	39.0	5.1	146.2	117.8	70	16	2000 dog units suprarenal cortical extract
Control	3	13.5	555	86.6	86.7	648.0	12.9	0.483	0.974	6.8	15.0	46.0	5.3	146.7	114.3	72	24	Normal saline solution

* This dog received 450 gm. of ground, mixed, raw beef, 3 cc. cod liver oil, 0.5 gm. of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, 500 cc. water, and 4 gm. of NaCl (by stomach tube) daily throughout the course of the experiment.

experiment large quantities of hormone (1000 dog units injected subcutaneously twice daily) delayed the appearance of the rebound effect until the 2nd day following hormone administration.

In another series of experiments the hormone was injected subcutaneously twice daily for 5 days. The urinary excretion of electrolytes was determined for the 5 day period. A daily average was calculated (Table III). In this experiment the changes were qualitatively similar to those described above. There did not appear to be a cumulative action of the hormone. The continued injection of suprarenal cortical hormone was accompanied by a reduction in the concentration of the cellular constituents of the blood and the plasma proteins without a decrease in the plasma concentration of sodium and chloride.

TABLE IV

The Effect of a Single Intravenous Injection of Suprarenal Cortical Hormone on Renal Excretion of Sodium and Potassium in the Normal Dog*

Hour	Urine volume		Sodium		Potassium	
	Control	Treated	Control	Treated	Control	Treated
	cc.	cc.	m. eq.	m. eq.	m. eq.	m. eq.
9 a.m.-3 p.m.	67	61	4.0	1.8	0.4	1.7
3 p.m.-9 a.m.	250	270	50.9	47.4	20.3	23.9
24 hr. total. . .	317	331	54.9	49.2	20.7	25.6

* 1000 dog units of suprarenal cortical hormone were injected at 9 a.m.

Intravenous injections of large quantities of hormone (3000 dog units) in the thirsted and fasted dog were not accompanied by plasma dilution.

A single intravenous injection of 1000 dog units of suprarenal cortical hormone resulted in a decreased sodium excretion and an increase in potassium excretion (Table IV). The action of the hormone was most marked during the 6 hour period immediately following injection. A slight reduction occurred in the 24 hour excretion of sodium. In normal human subjects repeated intravenous injections of hormone (500 dog units four times daily) were shown to be quite effective in producing a significant reduction in the 24 hour renal excretion of sodium (9).

The effectiveness of oral administration of the hormone has been investigated. A preparation which is ordinarily given parenterally must be administered in much larger quantities, when given by mouth, to produce an equivalent effect on the sodium and potassium excretion in the normal dog.

Three types of control material were injected subcutaneously into dogs without producing a significant effect on the urinary excretion of

TABLE V

A Comparison of the Effect of Subcutaneous Injections of Suprarenal Cortical Hormone on the Renal Excretion of Electrolytes in the Normal Dog Fed Diets High and Low in Mineral Content*

Dog	Period	Meat diet				Cowgill diet (K-free salt mixture)			
		Urine volume per day	Sodium per day	Potassium per day	Inorganic phosphorus per day	Urine volume per day	Sodium per day	Potassium per day	Inorganic phosphorus per day
		cc.	m. eq.	m. eq.	mg.	cc.	m. eq.	m. eq.	mg.
1-70	Control	555	51.3	20.0		215	2.6	Q. N. S.	
	Treated	465	32.0	23.8		195	1.2	Q. N. S.	
	Control	605	58.7	17.2		195	2.7	Q. N. S.	
1-75	Control	480	37.8	21.2	484	210	1.4	Q. N. S.	531
	Treated	535	36.5	29.3	555	210	0.4	Q. N. S.	472
	Control	445	40.7	17.8	470	225	1.2	Q. N. S.	520
1-76	Control	680	65.6	23.6	693	498	3.9	Q. N. S.	283
	Treated	675	53.1	32.9	762	595	0.5	Q. N. S.	408
	Control	680	73.4	27.6	680	510	2.6	Q. N. S.	333

Q. N. S. denotes quantity insufficient for analysis.

* The extract was injected in divided doses twice daily (2000 dog units).

sodium and potassium: (a) sterile normal saline solution in quantities equal to the sodium and chloride content of the extracts; (b) a 1:600,000 solution of epinephrin;⁴ (c) suprarenal cortical extract, inactivated by the addition of alkali and boiling, and subsequently brought back to the original hydrogen ion concentration.

The effect of the hormone was compared in three normal dogs given

⁴ This very dilute solution of epinephrin, representing the maximum present in any of the extracts, was freshly prepared immediately before injection.

diets of high and low mineral content (Table V). The dogs were studied first on a meat diet supplemented with sodium chloride, and secondly on the synthetic diet described by Cowgill (10). The latter was modified so as to contain no potassium. Although the sodium excretion was very greatly reduced during the period in which the animals received the modified Cowgill diet, injections of the hormone resulted in a measurable decrease in the amount of sodium excreted. The reduction in sodium excretion and the increased phosphate excretion were similar to the effect noted during the period in which the dogs were being maintained on a raw meat diet supplemented with sodium chloride. The action of the hormone on the renal excretion of sodium was demonstrable in the normal dog even though the potassium exchange was so low as not to be measurable.

Assay of Salt and Water Hormone

An important obstacle to the isolation and chemical identification of the suprarenal cortical hormone has been the lack of a reliable and expeditious method of assay. In addition to the fundamental observation of survival of the completely suprarenalectomized animal, three principal methods have been proposed. These are as follows: (a) maintenance of normal growth or survival in the suprarenalectomized rat (11); (b) maintenance of blood non-protein or urea nitrogen level in the suprarenalectomized dog (8); and (c) the protection of rat muscle against fatigue by means of suprarenal extract injections (12). The progress of investigation upon the suprarenal cortex during the past 6 years has indicated plainly that at least three hormonal principles may be present. One of these is concerned with sex development, a second with the metabolism of protein, and a third with the metabolism of sodium and potassium. It is the latter hormone which appears to be necessary, at least in the absence of an adequate sodium intake, to sustain life.

The regular response of the normal dog to subcutaneous injection of suprarenal cortical hormone suggests the possibility of measuring the renal excretion of sodium as a method of assaying the "salt and water" hormone content of suprarenal cortical extracts. Such a method would seem to be a rational procedure as it is now well established that this is a physiological effect of the suprarenal cortical hormone. As far as can be determined, the effect of a given extract on sodium excretion

in the normal dog, parallels the potency of the extract as determined by its effect on suprarenalectomized dogs. However, it is evident that other substances may be found which have a similar effect to that demonstrated for the cortical hormone upon the renal excretion of sodium and potassium.

The proposed method of assay is as follows: Under the standardized conditions described above, the maximum variation in the daily renal

TABLE VI

The Effect of Various Quantities of Suprarenal Cortical Hormone (Quantitative Assay) on Renal Excretion of Sodium and Potassium in the Normal Dog

Day	Urine volume per day	Sodium per day	Potassium per day	Quantity of extract
	cc.	m. eq.	m. eq.	
Control	1230	58.8	21.7	8 cc. extract A
Treated	1110	38.6	33.1	
Control	1210	76.0	26.5	
Control	1108	59.0	18.4	4 cc. extract A
Treated	1180	49.0	21.0	
Control	1230	69.2	14.0	
Control	1060	63.4	17.5	2 cc. extract A
Treated	1290	55.4	23.6	
Control	1340	67.0	19.4	
Control	1000	54.0	13.9	2 cc. extract B
Treated	1290	41.2	26.4	
Control	1250	62.6	13.1	
Control	1154	57.2	23.4	1 cc. extract B
Treated	1180	47.0	29.2	
Control	1206	59.4	22.2	

Extracts A and B were commercial extracts prepared by different companies. The assay value on suprarenalectomized dogs was the same. The extract was injected subcutaneously in divided doses, twice daily.

excretion of sodium is less than 5 m. eq. A reduction in sodium excretion greater than 5 m. eq., is significant, particularly if the control period following extract administration reflects a rebound in the sodium excretion. The relative potency of various extracts may be measured by determining the minimal amount of extract necessary to produce a 15 to 20 per cent reduction in the 24 hour renal excretion of sodium. The extract is injected subcutaneously in equal doses

twice daily. Normal dogs vary in the absolute amount of hormone necessary to show this change, but the results obtained on any given animal are reproducible. A similar individual variation has also been observed in suprarenalectomized dogs.

It is likely that the suprarenalectomized dog would prove to be a more sensitive preparation for assay. This, however, would require maintenance at a constant level of sodium excretion which would undoubtedly present serious difficulties. Either the animal would have to be maintained on sodium salts alone or a minimal ration of extract would have to be given. For this reason we have not attempted to use this method of assay on suprarenalectomized dogs.

SUMMARY

The effect of the administration of suprarenal cortical hormone upon the excretion of sodium and potassium has been studied on normal dogs. It is shown to be qualitatively similar to the effect of this hormone upon suprarenalectomized animals and upon patients with Addison's disease. The phenomenon is proposed as the basis for a suitable method for comparative assay of suprarenal cortical extracts. The method is simple, time saving, and uses a minimum of extract.

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THE EFFECT OF PROLONGED CULTIVATION IN VITRO UPON THE PATHOGENICITY OF YELLOW FEVER VIRUS

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All strains of yellow fever virus so far studied (about thirty in number) exhibit two major tissue affinities, namely, viscerotropic and neurotropic. By viscerotropic affinity is meant that affinity which is responsible for the classical lesions of yellow fever. The organ chiefly attacked is the liver. The experimental animal generally used to demonstrate this affinity is the *rhesus* monkey (1). The final outcome of an infection in the monkey depends upon the virulence of the virus. Highly virulent viscerotropic strains produce death in most of the animals. The majority of strains of yellow fever virus isolated from human cases exhibit a mild degree of virulence for the *rhesus* monkey and seldom produce death. That such monkeys undergo an infection can readily be shown by the demonstration of virus in the blood.

To demonstrate this, advantage is taken of the neurotropic affinity of the virus (2). Mice are inoculated intracerebrally with serum obtained from the infected monkeys at regular intervals after inoculation (3). Presence of virus is shown by the development of a fatal encephalitis in the inoculated mice. This method is sufficiently accurate and sensitive to allow a quantitative estimation of the virus in the circulating blood. Numerous studies along these lines have clearly shown that the quantitative study of virus in the circulation gives a reasonably accurate index of the viscerotropic affinity of the virus. The more virulent the virus the higher its concentration in the circulating blood during infection. By appropriate methods the viscerotropic affinity of the virus can be diminished. This can be most readily achieved by serial propagation of the virus in mouse brains (2, 4). This procedure, however, not only reduces the viscerotropic properties but also enhances the neurotropic affinities (5, 6).

Through serial passages by direct intrahepatic inoculation of *rhesus* monkeys, Findlay and Clarke (7) state that they have restored the viscerotropic affinity which had been lost through prolonged mouse brain passages. It would appear, therefore, that the inherent tissue affinities of yellow fever virus can be increased or decreased by appropriate procedures. This was clearly demonstrated by Lloyd, Theiler, and Ricci (8) when they employed tissue culture methods for determining the effect of prolonged cultivation in various types of tissues upon the yellow fever virus. The tissues chiefly used in their studies were minced chick

and mouse embryos, and minced testicles of guinea pigs and mice. Their results indicated that a prolonged cultivation *in vitro* of a highly virulent strain of the virus had resulted in a marked loss of the power to produce a fatal infection with typical visceral lesions in monkeys. On the other hand, there was no evidence to indicate that the degree of neurotropism of the virus had increased during cultivation in the various tissue media. Most of the experiments reported by these authors were carried out with virus grown in mouse embryo tissues. Although there was no clear evidence to indicate that the virus grown in one type of tissues had undergone much greater change than in another, certain suggestive differences were noticeable.

The purpose of the present paper is to report the changes that have taken place in yellow fever virus in the course of continued cultivation *in vitro* for a period of over 3 years without intervening passage through a living host. These observations were made upon the changes induced in the virus cultivated in media containing three different types of tissues. As the three different series of cultures were all derived from the same original virus, it would seem reasonable to conclude that the changes in the pathogenicity and the tissue affinity that occurred during the prolonged cultivation are in all probability attributable to the different cellular components of the tissue culture media. This work is essentially the continuation of that reported by Lloyd, Theiler, and Ricci (8), and the present authors owe a deep sense of gratitude to the late Dr. Wray Lloyd, through whose untiring efforts the success of initiating these experiments is largely due, and regret that by his untimely death he was unable to see the fruits of his labors.

Methods and Materials

Strains of Virus.—The strain of virus used to initiate these series of tissue culture experiments was the so called Asibi strain (1). The history of this strain from the time of its isolation from man to adaptation and growth in tissue culture has already been reported in full (8). It was first successfully established in tissue culture containing mouse embryonic tissue and 10 per cent normal monkey serum in Tyrode solution. Up to the time of writing it has been maintained without interruption in this medium for more than 240 subcultures during a period of over 3 years. For the purpose of reference, this strain is designated as 17 E.

After cultivation through eighteen subcultures in this medium, a separate branch was initiated from this virus in a medium containing minced whole chick embryo. After further cultivation through 58 subcultures in the latter medium, the tissue component was modified to the extent that instead of using the whole

minced chick embryo, the brain and spinal cord were removed from the embryo before mincing. The virus has been maintained continuously in this medium containing minimal amounts of nervous tissue for over 160 subcultures. This strain is designated as 17 D. The third strain of virus included in this study and referred to as 17 AT was obtained from 17 E after 27 subcultures. The tissue component of the medium used for the cultivation of this series was minced adult mouse testicle. After 70 subcultures in this medium, adult guinea pig testicle was substituted for the mouse testicular tissue. The virus was grown for another 90 subcultures in this medium, after which the experiment was discontinued.

Methods of Cultivation.—The methods of virus cultivation used have been fully described previously and are essentially similar to the technique used by Rivers (9) and Rivers and Ward (10) for the propagation of vaccinia virus *in vitro*. The culture medium generally consisted of minced tissues and Tyrode solution containing 10 per cent of normal monkey or human serum. The cultures were incubated at 37°C. and subcultures were made every 3 or 4 days. At every passage the infectivity of the supernatant fluid of the centrifuged tissue culture was tested by the intracerebral injection of mice. At regular intervals portions of the culture virus were desiccated in vacuum in the frozen state for the purpose of preservation (11).

Titration of Virus and Antibody.—Mice of susceptible strains were used for titrating virus preparations. Groups of at least six mice were inoculated intracerebrally with 0.03 cc. of decimal dilutions. From the resulting mortality the theoretical dilution which would produce a 50 per cent mortality was determined statistically by Muench's method (12). In titrating the antibody content, the intracerebral protection test was used (13). This method consists essentially of mixing serial dilutions of the serum with a standard amount of virus, incubating the mixture for 1 hour at 37°C., and inoculating groups of mice with each mixture. From the resulting mortality in mice, the theoretical dilution of serum which would protect one-half of the mice is determined. Each protection test is controlled by the titration of a known immune serum. The titer of a serum is expressed as that theoretical dilution of the serum which, under the conditions of the test, protects one-half of the mice inoculated.

Tests for Immunity.—The immunity of all monkeys which survived inoculation with tissue culture was tested by two procedures. In almost all animals the antibody titer of the serum 1 month after inoculation was determined by means of the intracerebral protection test in mice. In addition, the actual immunity of the monkeys was tested by the injection of a highly virulent virus. In the great majority of instances the virus used for this purpose was virulent Asibi strain, which was injected intraperitoneally. This virus produces a mortality from visceral lesions in about 95 per cent of normal monkeys. The immunity of some monkeys was tested by the intracerebral inoculation of the French neurotropic virus. This virus variant was produced by the serial passage of the so called French strain (2) through more than 200 mice by direct brain to brain transmission. When inoculated intracerebrally, this virus invariably produces death from encephalitis in normal monkeys (6).

Tests for Circulating Virus.—There is a distinct correlation between the amount of circulating virus in the blood of a monkey and the virulence of the virus. The titer of the virus in the circulating blood is highest in those animals which die of viscerotropic lesions (3). Consequently, the duration and the relative concentration of the virus in the peripheral blood of monkeys during infection serve as an index of the viscerotropic affinity of the virus. The presence of virus in the blood of monkeys is determined by intracerebral inoculation of mice with the serum of monkeys obtained at regular intervals after infection. In the studies reported here, no effort was made to determine the titer of the virus in the peripheral circulation, and only its presence or absence was tested.

Pathogenicity of the Cultivated Strains of Yellow Fever Virus for Mice

As a rule at each subculture mice were inoculated intracerebrally with the supernatant fluid used for making a transfer to fresh medium and thus the presence of virus in the cultures was verified. The impression was obtained from the incubation period and time of death of the mice that there was a marked difference in the same strain of yellow fever virus grown for prolonged periods of time in various media. In general, mice inoculated with virus grown in chick embryo tissue exhibited a longer incubation period and a longer period of sickness than mice inoculated with virus grown in mouse embryonic tissue. As the incubation period in mice inoculated intracerebrally depends not only on the strain of virus used but also on its concentration, the results of numerous titrations were analyzed. These showed quite clearly that the virus grown in chick embryo tissue was less neurotropic for mice than that cultivated in mouse embryonic tissue.

Another index of neurotropism available is the rate at which a virus becomes fixed when passed serially through a number of mice by intracerebral inoculation. Such serial passages were initiated with the virus grown in three different types of tissue culture media as well as with the unmodified Asibi strain which, it will be recalled, is the parent strain from which the tissue culture series were branched off. The subcultures used to initiate the serial passages in mice were the 176th subculture of the virus 17 D grown in chick embryo, the 157th subculture of the virus 17 E grown in mouse embryo, and the 160th subculture of the virus 17 AT grown in testicular tissue.

Six to eight mice were used for each passage and the time of death was noted. The average time of death in the mice inoculated at each subsequent passage with the different viruses was calculated, and a

statistical analysis of the figures was made.¹ All four series of mouse passages showed a progressive shortening of the time from inoculation to death. The rate of fixation varied considerably, however. It was greatest with the parent Asibi strain and lowest with virus 17 D grown in chick embryo tissue.

Several points of interest were brought out in this analysis. The first was that there was no marked difference between the parent strain and the strain grown in mouse embryo tissue for 157 subcultures. The second was that the strain of virus grown for 160 subcultures in testicular tissues and the strain maintained for 176 subcultures in chick embryo tissue were significantly less neurotropic than the parent Asibi strain. At the time that this serial passage in mice was commenced, the virus grown in chick embryo (17 D) had been cultivated through 176 subcultures in a medium containing chick embryo tissue. During the last 100 of these subcultures only minimal amounts of nervous tissue were incorporated in the medium. It seems hardly a coincidence that the two least neurotropic viruses were obtained in media containing also the least amounts of nervous tissue.

In order to obtain information as to the time when the loss of neurotropicism for mice appeared in the virus 17 D, serial passages in mice were begun with its 114th subculture. At this time the virus had been maintained for 96 subcultures in the chick tissue and during the last 38 subcultures only minimal amounts of nervous tissue were present in the medium. Although at the time of writing only 15 serial passages have been made with this virus, the results nevertheless suggest that the virus at this stage of cultivation is far more neurotropic than it was in the 176th subculture. The average time of death in the first 15 passages in mice with this virus in the 114th and 176th subcultures is shown in Table I. The average time of death of mice inoculated in series with virus from the 176th subculture is significantly later than that of those inoculated in series commencing with the 114th subculture. This loss of neurotropicism for mice is evidently progressive in a medium containing chick embryonic tissue, and as will be shown below, the virus grown in this medium loses its power to produce fatal encephalitis in monkeys between the 89th and 114th subcultures.

¹ This analysis was kindly made for us by Dr. H. Muench.

*Pathogenicity of Cultivated Strains of Virus for Rhesus Monkeys**Virus 17 E Grown in a Medium Containing Mouse Embryo Tissue.*—

In the previous communication by Lloyd, Theiler, and Ricci (8), the results of inoculating *rhesus* monkeys intraspinally and intracerebrally with virus grown in the presence of mouse embryo tissue have been presented. The supernatant fluid from the 35th to the 82nd subculture

TABLE I

Average time of death in mice infected with virus 17 D grown in chick embryo tissues; mice inoculated with 114th and 176th subcultures, and subsequent passages made in two parallel series by means of intracerebral inoculation of infected brain suspension.

No. of serial passage in mice	Average time interval between inoculation and death			
	Series initiated from 114th subculture		Series initiated from 176th subculture	
	Average for each passage	Average for 5 passages	Average for each passage	Average for 5 passages
	<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>
2	7.5		9.1	
3	6.8		10.1	
4	6.7		10.0	
5	7.7	7.1	9.7	9.8
6	8.5		9.7	
7	7.0		10.4	
8	7.9		9.3	
9	7.4		9.0	
10	7.5	7.0	10.4	9.6
11	6.3		10.8	
12	6.8		9.1	
13	7.4		8.2	
14	7.4		8.2	
15	7.8	7.2	8.0	9.1

was used. Three monkeys inoculated intracerebrally died presenting the histopathological lesions of encephalitis. Two of them also showed hepatic necrosis typical of visceral yellow fever. One of the latter had been inoculated with virus from the 82nd subculture. Ten monkeys were inoculated intraspinally with material from the 41st to the 76th subculture. All ten animals showed a febrile reaction. Three monkeys died, two presenting lesions of visceral yellow fever

and one of encephalitis. In six monkeys the circulating blood was examined for virus, which was shown to be present in all for a period of from 3 to 5 days. The significant features of these animal experiments were the production of visceral lesions of yellow fever even after 82 subcultures, the presence of circulating virus in the blood of all animals examined, and the relatively few animals which died of encephalitis following intraspinal injection of virus.

TABLE II

Results of Intracerebral Inoculation into Rhesus Monkeys of Virus 17 E Grown in Mouse Embryo Tissue Cultures

Monkey No.	Inoculum		Test for virus in circulating blood; mortality ratio in mice inoculated with serum*							Fever on days after inoculation	Time to death after inoculation*	Mortality ratio in mice inoculated with brain suspension*	Histopathology of brain
	Subculture	Amount	Days after inoculation										
			1	2	3	4	5	6	7				
1	201	1.0 cc.	6/6		4/7		0/4		3, 4, 5, 7	10	5/6	Encephalitis	
2	"	"	4/5		7/7		0/7		4, 5, 6, 7	9	7/7	"	
3	203	"	7/7	7/7		3/6	0/6	6, 7, 8	12	Not tested	"		
4	"	"	3/6	7/7		4/7	0/6	3, 4, 7	9	4/6	Not examined		
5	"	"	4/7	5/6		3/5	1/6	5, 6, 7, 8	11	4/6	Encephalitis		
6	"	"	5/7	7/7		4/6	0/6	3, 4, 5, 6, 7	10	5/5	Appears normal		
7	"	"	3/6	6/7		1/6	0/5	6, 7, 8	10	5/6	Encephalitis		

* In all tables the numerator represents the number of mice that succumbed to infection; the denominator, the number of mice used in the test.

In the present study the neurotropism of the cultivated strain of virus for *rhesus* monkeys was tested by the far more severe test of intracerebral inoculation. In all, seven monkeys were inoculated intracerebrally, two with the supernatant fluid from the 201st subculture, and five with the supernatant fluid from the 203rd subculture. The results are shown in Table II. All the monkeys responded with a febrile reaction commencing on the 3rd to the 6th days after inoculation and lasting for 3 to 5 days. In their blood circulating virus was shown to be present for 3 to 4 days, and all the animals died in from

9 to 12 days after inoculation. The diagnosis of yellow fever virus encephalitis was confirmed in all the animals by histopathological study or by isolation of the virus from their brains after death, or by both methods. In all the animals the virus had disappeared from the blood at the time of death.

These studies give no indication of an apparent loss of neurotropism for monkeys of the virus cultivated in mouse embryo tissue. It must be emphasized that the strain of yellow fever virus used to initiate the culture is not only highly viscerotropic but also neurotropic to a high degree. This latter quality in the unmodified virus can be readily demonstrated in the *rhesus* monkey by intracerebral inoculation of the virus if the animal is given simultaneously an injection of immune serum intraperitoneally. Under these conditions the monkey develops a fatal encephalitis.

Virus 17 AT Grown in Testicular Tissue.—The amount of experimental evidence available concerning the affinity of the virus grown in testicular tissue for the central nervous system of monkeys is meagre. Lloyd, Theiler, and Ricci (8) reported the results obtained on inoculating four monkeys intraspinaly with virus from the 30th and 35th subculture in testicular tissue. This virus had previously been maintained for 27 subcultures in mouse embryo tissue. All four monkeys died of yellow fever virus encephalitis in from 10 to 13 days. Noteworthy is the fact that in three of these monkeys, which were studied for the presence of virus in the blood, only minimal amounts could be demonstrated.

In the present study the pathogenicity for *rhesus* monkeys on intracerebral inoculation of the virus grown in testicular tissue was tested with the 90th subculture in guinea pig testicular tissue. This virus had been in tissue cultures for a total of 187 passages, during the last 160 of which testicular tissue was used. Two monkeys were inoculated intracerebrally with 1.0 cc. amounts of supernatant fluid. The results are shown in Table III. Both monkeys died of encephalitis, one on the 9th and the other on the 12th day following inoculation. In the blood of both only small amounts of circulating virus were demonstrated.

Virus 17 D Grown in Chick Embryo Tissue.—Three sets of observations are available concerning the pathogenicity of the virus grown in chick embryo tissue. The first experiment was performed with the 89th subculture, and has been previously presented (8). This experiment is included here for the sake of completeness. In this test six monkeys were inoculated intraspinaly and three of them, Nos. 13, 14, and 15, received in addition 2.0 cc. of the culture intraperitoneally (see Table IV). All six animals died of encephalitis in 10 to 13 days after inoculation. Virus was shown to be present in the blood of all during a period of from 3 to 6 days.

The second experiment deals with four *rhesus* monkeys inoculated intracerebrally with virus from the 114th subculture. The concentration of virus in tissue culture is not very great. In order, therefore, to use a greater amount of virus for the inoculation of monkeys, several mice were inoculated intracerebrally with rehydrated material representing the 114th subculture. When these mice became sick as a result of yellow fever virus encephalitis, they were killed and from their brains a 10 per cent suspension was made. Four monkeys were inoculated intracerebrally with 1.0 cc. of the suspension. Titration of the suspension in mice showed that each monkey received approximately two million average lethal doses for mice. The results are shown in Table IV. All four monkeys lived, and the blood of two was followed for the presence of circulating virus. In the blood of one animal very small amounts were demonstrated on the 2nd and 3rd days; whereas in the blood of the other sufficient virus was present on the 2nd day fol-

TABLE III

Results of Intracerebral Inoculation into Rhesus Monkeys of Virus 17 AT Cultivated in Testicular Tissue Medium

Monkey No.	Inoculum		Test for virus in circulating blood: mortality ratio in mice inoculated with serum				Fever on days after inoculation	Time to death after inoculation	Mortality ratio in mice inoculated with brain suspension	Histopathology of brain
	Subculture	Amount	Days after inoculation							
			3	5	7	9				
8	160	1.0	0/6	3/6	1/7	0/5	4, 5, 6, 7	9	5/6	Encephalitis
9	160	1.0	0/5	2/6	0/6	0/5	Continuous 3rd to 10th	12	6/7	"

lowing inoculation to kill all of the five mice. Neutralizing antibodies were demonstrated in the serum of the four monkeys 1 month after inoculation. Their immunity was tested by an intracerebral inoculation of the most highly neurotropic yellow fever virus. All four monkeys lived, though one responded with a fever. It may be noted that this animal had the lowest antibody titer of the four monkeys in its serum at the time of the immunity test.

The third series of monkeys consisted of seven inoculated with material representing the 186th to the 216th subcultures in a medium containing chick embryo tissue. All responded with febrile reaction. Two of the monkeys died of intercurrent disease, No. 21 from peritonitis on the 29th day and No. 25 from tuberculosis on the 17th day. The brain of the latter, though free of virus as tested by intracerebral inoculation of mice, nevertheless showed histopathological signs of encephalitis in the form of a very mild perivascular infiltration. This histopathological evidence, as well as the febrile reaction in all the monkeys following

CULTIVATION OF YELLOW FEVER VIRUS

Monkey No.	Sub-culture used for inoculation	Route	Test for virus in circulating blood: mortality ratio in mice inoculated with serum									Fever on days after inoculation	Time to death after inoculation	Antibody titer 4 wks after inoculation	Result of immunity test	
			Days after inoculation												Asibi virus subcutaneously	French neurotropic virus intracerebrally
			1	2	3	4	5	6	7	8	9					
10	89th	i.s.		3/6		5/5			2/6			7, 8	10			
11	"	"		4/6	4/5		4/4		5/6			4, 5, 6, 7, 8	13			
12	"	"		5/6		5/6		5/6				6, 7, 8	11			
13	"	i.s. and i.p.		5/6	6/6		3/3		4/6			Continuous to 10th	13			
14	"	"		6/6		5/5		3/6				1, 3, 5, 6, 7	12			
15	"	"		5/6	5/5		5/5		5/5			—	10	1:2		Lived
16	114th	i.c.										2, 3, 4, 6, 7, 9, 10	Lived	1:3		"
17	"	"										8	"	1:4		"
18	"	"		0/6	2/6	2/6	0/2	0/3	0/6	0/4	0/6	Continuous to 10th	"	"		"
19	"	"		0/6	5/5	3/6	0/4	0/6	0/5	0/6	0/6	7, 8, 9	"	1:2		
20	186th	"										1, 2	"	1:28		Lived
21	"	"			0/5		1/6		2/5		0/5	Continuous to 10th	"	"		
22	"	"										8, 9, 10	"	1:25		Lived
23	216th	"			0/5		0/6		2/7		0/6	6, 7, 8, 9	"	Positive not titrated		
24	"	"										Continuous to 10th	"	"		
25	"	"										6, 7	†	"		
26	"	"										6, 7, 8, 9, 10	Lived	"		

i.s., intraspinally. i.p., intraperitoneally. i.c., intracerebrally. s.c., subcutaneously.

* Died of peritonitis 29 days after inoculation. † Died of tuberculosis 17 days after inoculation.

the intracerebral inoculation of the virus grown in chick embryo tissue, on the one hand, and the absence of febrile reaction when the same virus was injected subcutaneously, on the other hand, clearly indicate that the virus is capable of producing a mild non-fatal encephalitis.

The remaining five animals showed protective antibodies in their serum obtained 4 weeks after inoculation. Two of the monkeys, Nos. 20 and 22, were given a test dose of highly virulent viscerotropic Asibi virus subcutaneously and showed no reaction.

From these three experiments it is evident that a marked change was induced in the virus grown in chick embryo tissue. This change had a comparatively sudden onset. The virus from the 89th subculture was highly neurotropic for monkeys and still had the ability to produce a visceral infection characterized by an abundance of virus in the circulating blood. The virus in the 114th subculture had lost both of these qualities.

Extraneural Inoculation of Rhesus Monkeys with Cultivated Strains of Virus

Virus 17 E Cultivated in Mouse Embryo Tissue.—The progressive loss of the power of the pantropic yellow fever virus grown in mouse embryo tissue to produce death in monkeys has been reported in full previously (8). Of seventeen monkeys inoculated intraperitoneally or subcutaneously with supernatant fluid from the 49th to the 109th subculture, fourteen responded with a febrile reaction of 1 to 5 days' duration. All survived. Virus was consistently demonstrated in the blood of all the animals on 2 or more days during the first week following inoculation.

In the present study the results of inoculating ten monkeys with material from the 179th and 206th subcultures are shown in Table V. In this experiment emphasis was placed on the presence of circulating virus, as it affords a far more delicate index of the viscerotropic affinity of a virus. Examination of the table shows that virus was present for 3 to 7 days in each animal. Of the ten monkeys, only No. 30 had a febrile reaction which could be attributed to the inoculated virus. Three monkeys, Nos. 27, 28, and 35, had irregular fever; in Nos. 27 and 28, it was attributed to tuberculosis, as both showed extensive tuberculous lesions when submitted to autopsy at the termination of the experiment. Monkey No. 33 died of intercurrent disease, and the remainder lived and were shown to be immune when tested by inoculation of virulent yellow fever virus 34 to 42 days after the injection of the tissue culture virus.

Virus 17 AT Cultivated in Testicular Tissue.—No specific experiments were undertaken to determine the pathogenicity of this strain of virus for monkeys by extraneural inoculation. From previous work and also the study of circulating virus in monkeys inoculated intracerebrally, the conclusion seems justified that

TABLE V
Results of Inoculation of Virus 17 E Grown in Mouse Embryo Tissue Cultures into Rhesus Monkeys Subcutaneously

Monkey No.	Inoculum		Test for virus in circulating blood; mortality ratio in mice inoculated with monkey serum										Fever on days after inoculation	Result	Antibody titer 30 days after inoculation	Test for immunity with Asibi virus
			Days after inoculation													
	Sub-culture	Amount	1	2	3	4	5	6	7	8	9	10				
27	179th	cc.	1/6	4/4	10/11	10/12	9/9	8/12	4/11	0/12			Irregular	Lived	Not tested	Lived
28	"	"	0/4	1/4	9/11	10/11	6/8	12/12	2/12	1/11			"	"	"	"
29	"	"	0/4	1/4	10/11	11/12	11/12	6/12	4/12	2/12			—	"	"	"
30	"	"	0/6	4/4	7/9	9/12	7/10	7/10	2/12	0/12			4, 5, 7	"	"	"
31	"	"	0/6	1/3	9/11	10/10	7/9	7/11	2/12	0/13			2	"	"	"
32	"	"	0/4	1/4	7/8	10/11	10/12	8/12	2/12	0/12			—	"	"	"
33	206th	1.0	0/5	1/5	6/7	6/6	6/6	3/3	3/7				—	*		"
34	"	"	2/5	3/4	5/6	2/5	2/6	0/3	0/6				—	Lived	1:110	"
35	"	"	0/5	0/4	4/5	6/6	6/7	3/4	0/6				Irregular	"	1:38	"
36	"	"	0/5	0/5	5/6	5/5	4/6	0/4	0/7				—	"	1:45	"

* Died of colitis 14 days after inoculation.

this virus has a tendency to produce mild systemic infections with only minimal amounts of virus in the circulating blood.

Virus 17 D Cultivated in Chick Embryo Tissue.—Data on three groups of monkeys inoculated extraneurally with virus cultivated in chick embryo tissue are presented. The first group consists of three monkeys inoculated intraperitoneally with 2.0 cc. of supernatant fluid from the 89th subculture. Virus was shown to be present in the circulating blood of two of these animals for a duration of 5 and 6 days. The third animal was not studied from this point of view, though on one occasion when mice were inoculated intracerebrally with its serum, virus was shown to be present. Monkey 38 responded with a febrile reaction of 5 days' duration beginning the 3rd day after inoculation. This animal died on the 16th day and at autopsy showed lesions of tuberculosis. Histopathological studies, however, revealed encephalitis also. The other two animals remained well and resisted a test dose of virulent Asiatic virus.

The second group of animals consists of four monkeys inoculated subcutaneously with material representing the 114th subculture of the virus in chick embryo tissue. The inoculum consisted of 1 in 20 suspension of infective mouse brains prepared from the brains of mice sick after an intracerebral inoculation of the 114th subculture. The same material was used to inoculate four monkeys intracerebrally, the results of which have been previously mentioned (monkeys 16 to 19, Table IV). Titration in mice by intracerebral inoculation of decimal dilutions showed that each monkey received approximately one million average lethal doses of virus for mice. All four monkeys were bled daily for the first 10 days from the vein, and the serum tested in mice for presence of virus. It will be seen from a study of Table VI that virus was shown to be present in the blood of three animals, though only in minimal amounts, the serum in no instance having sufficient concentration of virus to kill more than one mouse of the group inoculated. Protection tests with the sera taken 7 and 14 days after inoculation show that at 7 days antibodies were beginning to be present and were well developed by 14 days. All four animals survived. Their immunity was tested 41 days after the original inoculation by intracerebral injection of the French neurotropic virus. The four monkeys responded to this test inoculation with a febrile reaction commencing on the 4th and 6th days and lasting from 3 to 5 days. All lived, though No. 43 showed signs of encephalitis on the 8th day following the test inoculation.

The third set of observations concerns nine monkeys, of which four were inoculated with 0.5 cc. and five with 1.0 cc. of the supernatant fluid from the 186th, 195th, and 197th subcultures. Monkey 48 responded with a febrile reaction of 1 day's duration on the 5th day. Virus was proved to be present in the blood of five of these animals, although in two (Nos. 44 and 49) in very small amounts as only one mouse in a group became ill. In one animal, No. 50, virus was shown to be present for 5 days commencing on the 5th day after inoculation. Another monkey, No. 47, showed presence of virus on the 6th and 7th days in fair amounts, the last 2 days on which this animal's blood was tested. Monkeys 47 and 50 died

TABLE VI

Results of Inoculation of Virus 17 D Grown in Chick Embryo Tissue Culture into Rhesus Monkeys by Extraneural Routes

Non-key No.	Inoculum		Test for virus in circulating blood: mortality ratio in mice inoculated with serum										Fever on days after inoculation	Result	Antibody titer 4 wks. after inoculation	Result of immunity test		
	Sub-culture	Amount	Route	Days after inoculation												Asibi virus subcutaneously	French neuro-tropic virus intra-cerebrally	
				1	2	3	4	5	6	7	8	9						10
37	89th	cc.		4/6		6/6				2/6			—	Lived		Lived	Lived	
38	"	2.0		6/6									Continuous 3rd to 7th					
39	"	"		5/5	4/4					4/4		2/5	—	Lived				
40	114th	Each received about 1,000,000 mouse M.L.D.		0/4	1/6	0/5	0/4	0/6	0/6	0/5	0/5	0/6	2	"	1:10	Lived	Lived	
41	"	"		0/6	0/5	0/6	0/5	0/4	0/5	0/6	0/6	0/7	2	"	1:45	"	"	
42	"	"		0/5	0/5	0/5	1/4	0/5	0/6	0/6	0/3	0/5	2	"	1:28	"	"	
43	"	"		0/6	0/6	1/6	1/4	0/4	0/4	0/5	0/3	0/8	2	"	1:23	"	"	
44	186th	1.0		1/6	0/6	0/6							—	"	1:40	Lived		
45	195th	"		0/5	0/5	0/5	1/7	1/6	0/6	0/5			—	"	1:8	"		
46	"	"		0/4	0/6	0/4	1/7	0/6	1/6	2/4			—	"	1:54	"		
47	"	"		0/4	0/5	0/5	0/5	0/5	4/6	4/5			—	†				
48	"	"		0/6	0/6	0/4	1/6	0/6	0/6	0/2			5	Lived	1:16	"		
49	197th	0.5		0/6	1/6	0/7	0/7	0/6	0/8	0/7	0/6	0/8	—	"	Positive, not titrated	"		
50	"	"		0/6	0/8	0/8	0/8	5/7	5/6	8/8	4/7	2/8	0/8	†				
51	"	"		0/7	0/7	0/8	0/8	0/7	0/6	0/8	0/7	0/8	—	Lived	"	"		
52	"	"		0/7	0/7	0/7	0/8	0/7	0/7	1/8	5/6	2/8	0/8	"	"	"		

Underlined mouse groups show instances where death from yellow fever was verified.

* Monkey 38 died 16 days after inoculation showing tuberculosis as well as lesions of yellow fever.

† Monkey 47 died of colitis 10 days after inoculation and showed no evidence of yellow fever.

‡ Monkey 50 died of colitis 26 days after inoculation and showed no evidence of yellow fever.

of intercurrent diseases. The remainder lived, and all were shown to have developed neutralizing antibodies and to be immune to a test dose of virulent virus 36 to 37 days after the original inoculation.

Pathogenicity of the Cultivated Strains of Virus for Hedgehogs

Findlay and Clarke (14, 15) made the observation that the common European hedgehog is susceptible to the virus of yellow fever. Not only do these animals die after an inoculation with virulent viscerotropic strains of yellow fever virus but they also succumb to the infection after subcutaneous inoculation of modified neurotropic French strain. This latter strain, the character of which will be discussed in another paper (16), has been serially passed through a large number of mice by intracerebral inoculation and has lost its original viscerotropic affinity for monkeys to the extent that it rarely produces death with visceral lesions in them. Findlay showed, however, that in the hedgehog this virus still has the capacity to produce hepatic necrosis. It seems that the hedgehog therefore offers a more delicate means of determining the index of the viscerotropic potentialities of the virus than the *rhesus* monkey. It was decided to test the pathogenicity of the tissue culture virus in this species of animal. As the hedgehog has to be imported from Europe, the number of observations are limited.

Only two of the cultivated strains were tested in hedgehogs. They all were inoculated subcutaneously and the presence of virus in the liver and brain of those which died was tested by intracerebral inoculation of the organ emulsion in mice. The results are shown in Table VII. Of seven animals inoculated with virus 17 E grown in mouse embryonic tissue, No. 1 which had received virus from the 84th subculture died of yellow fever, whereas the six inoculated with the supernatant fluid from the 212th subculture all lived. The blood of these six hedgehogs was tested for the presence of neutralizing properties before inoculation and 25 days after they had received the injection of virus. The sera taken before the injection of virus were shown to be free of antibodies, whereas those obtained 25 days after inoculation showed the presence of antibodies.

The results in another series of six hedgehogs which were inoculated with the 198th subculture of virus 17 D grown in chick embryonic tissue were marred by the fact that five of the six animals died of intercurrent infection 12 to 14 days after inoculation. Intracerebral inoculation of mice with suspensions of liver and brain from these hedgehogs failed to reveal the presence of virus. An epidemic disease spread through all our hedgehogs at this time, and the mortality

among those under experiment was not higher than among those which were known to be immune to yellow fever.

In Table VII are also presented the results on eight normal hedgehogs inoculated subcutaneously with unmodified Asibi strain, the parent virus from which the tissue culture strains are derived. All eight animals died in 3 to 7 days. In the liver or brain, or both, of all the animals, virus was found to be present at death.

TABLE VII

Pathogenicity of Tissue Culture Strains of Yellow Fever Virus for Hedgehogs

Hedgehog No.	Inoculum		Result	Test for presence of virus at death	
	Virus	Subculture		Brain	Liver
1	17 E	84	Died of yellow fever	Positive	Positive
2	"	212	Lived		
3	"	"	"		
4	"	"	"		
5	"	"	"		
6	"	"	"		
7	"	"	"		
8	17 D	198	Died of intercurrent infection	Negative	Negative
9	"	"	Lived		
10	"	"	Died of intercurrent infection	Negative	Negative
11	"	"	" " " "		
12	"	"	" " " "		
13	"	"	" " " "		
14	Asibi, unmodified		Died on 7th day, yellow fever		
15	"	"	" " 7th " " "		
16	"	"	" " 3rd " " "		
17	"	"	" " 7th " " "		
18	"	"	" " 7th " " "		
19	"	"	" " 5th " " "		
20	"	"	" " 5th " " "		
21	"	"	" " 7th " " "	"	Negative

Though marred by the accidental deaths of five hedgehogs, the results give some definite information. It is apparent that after cultivation for 212 subcultures in mouse embryonic tissue the virus has lost its power to produce a fatal infection in hedgehogs. The results obtained with the virus 17 D grown in chick embryonic tissue can only be interpreted as indicating that this virus is avirulent for hedgehogs also. Although nearly all the animals had died of inter-

current disease, no virus could be demonstrated in the liver and brain at death and the conclusion seems warranted that no fatal infection followed inoculation. This conclusion is all the more probable when it is considered that in *rhesus* monkeys this virus has been shown to have less viscerotropic affinity than the variant grown in mouse embryonic tissue (17 E).

DISCUSSION

The results of these experiments show clearly that the modification induced in cultivated strains of yellow fever virus is determined by the nature of the tissue used in the medium. By prolonged cultivation in minced whole mouse embryonic tissue there is a progressive loss of the viscerotropic affinity of the virus but no obvious change in the neurotropic. Cultivation in testicular tissue induced a marked loss of viscerotropic affinity. Loss of neurotropic virulence was also induced but not to a marked extent. The loss of neurotropic affinity could be shown in mice but not in monkeys. Cultivation in a medium containing chick embryonic tissue induced a marked loss of both the viscerotropic and neurotropic affinities.

For a correct understanding of the changes induced, a clear knowledge of the pathogenicity of the virus before it was propagated in tissue culture is essential. The Asibi virus, the parent strain, is the most virulent yellow fever virus so far studied. When inoculated subcutaneously into monkeys, it produces death in 4 to 7 days in 95 per cent of these animals. Maximal amounts of virus are present in the blood. Not only does this virus exhibit a high viscerotropic affinity, but its neurotropic affinity is well marked, not only for mice but also for monkeys. Prolonged cultivation of the Asibi virus in a medium containing chick embryonic tissue leads to a marked loss of both of these two tissue affinities. Subcutaneous inoculation rarely produces febrile reaction and the quantity of virus demonstrable in the blood of infected monkeys is usually minimal. On intracerebral inoculation into monkeys, a non-fatal encephalitis is induced.

This marked loss of neurotropic affinity in the virus grown in minced chick embryonic tissue is in all probability due to the use of minimal amounts of nervous tissue in the medium. This was achieved by cutting away the brain and spinal cord of the chick embryos before

TABLE VIII
A Comparison of the Pathogenicity of Cultivated Yellow Fever for Rhesus Monkeys and Hedgehogs

Tissues used in culture medium	Results in monkeys		Results in hedgehogs by subcutaneous inoculation
	By extraneural inoculation	By intracerebral inoculation	
Whole mouse embryo—virus 17 E	Monkeys survive, but show a considerable amount of virus in the circulating blood.	Death from encephalitis	Survive
Mouse and guinea pig testicular tissue—virus 17 AT	Monkeys survive and show only traces of virus in the circulating blood	" "	Not tested
Chick embryo tissue with head and spinal cord removed—virus 17 D	Monkeys survive and show only traces of virus in the circulating blood	Non-fatal encephalitis	Survive
Unmodified Asibi virus	About 95 per cent of monkeys die of yellow fever showing typical visceral lesions; virus present in the circulating blood in high concentration	Death usually from generalized infection with typical visceral lesions. Death from encephalitis only when immune serum is given intraperitoneally at the time of intracerebral injection of virus	Death from yellow fever in 3 to 7 days with typical visceral lesions

mincing. It must be realized, however, that the experiments do not exclude the possibility that these marked changes were induced by the chick tissue itself and not by the relative absence of the nervous tissue. The pathogenicity of the three cultivated strains for monkeys and hedgehogs is summarized in Table VIII. Included in this table are the pathogenic characteristics of the Asibi strain of virus, the parent strain from which the three cultivated variants are derived.

SUMMARY

1. Experimental evidence is presented to show that prolonged cultivation of yellow fever virus *in vitro* results in a change in its pathogenicity, and that this change varies with the type of tissues used for the cultivation.

2. In the tissue cultures used for the propagation of the virus, three different types of tissues were used. They included whole mouse embryo, chick embryo from which the head and spinal cord had been removed, and testicular tissues of mice and guinea pigs.

3. The changes in the pathogenicity of the virus cultivated for a period of over 3 years in a medium containing the tissues of whole mouse embryo were not striking. The viscerotropic virulence of the virus appeared somewhat diminished, in that when injected subcutaneously into *rhesus* monkeys or hedgehogs it failed to produce a fatal infection, although there is evidence to indicate that a generalized infection takes place as demonstrated by the appearance of virus in the circulating blood in relatively high concentration during infection. The neurotropic virulence of the virus remained unaltered during the cultivation in this medium.

4. The changes in the pathogenicity of the virus cultivated in medium containing tissues of chick embryo from which the head and spinal cord had been removed were very pronounced. The viscerotropic virulence of the virus was lost to a large extent. When injected subcutaneously into monkeys there was as a rule a very mild generalized infection, as demonstrated by the minimal quantities of virus found in the circulating blood. Its neurotropism was also much diminished. When injected into monkeys intracerebrally, it no longer produced a fatal encephalitis but only a moderate febrile reaction, followed by recovery and solid immunity to reinoculation with a highly

virulent strain of virus. When injected intracerebrally into mice, the mortality ratio was not diminished but the incubation period was markedly prolonged.

5. The changes in the pathogenicity of the virus cultivated in medium containing testicular tissues were somewhat similar to those observed after cultivation in chick embryo medium which contained only a minimal amount of nervous tissue. Its viscerotropic affinity had been largely lost and only very small amounts of virus were found in the circulating blood of monkeys inoculated subcutaneously. Given intracerebrally, it produced death from encephalitis in monkeys. The incubation period in mice inoculated intracerebrally with this virus was also prolonged but somewhat less so than with the virus grown in chick embryo tissues without the central nervous system.

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THE USE OF YELLOW FEVER VIRUS MODIFIED BY IN VITRO CULTIVATION FOR HUMAN IMMUNIZATION

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One of the most striking phenomena to the student of virus diseases is the occurrence of variants. This phenomenon is of particular importance in that several such variants are being used for the immunization of man. The two classical examples of the use of attenuated forms of virus for human vaccination are vaccinia and the fixed virus of rabies. The origin of vaccinia virus is a moot point, but it is almost universally considered to be a variant of smallpox obtained by passage through the cow. This virus was found as such in nature. The fixed virus of rabies was produced by serial propagation in rabbit brain. By this procedure a variant was produced which had lost to a considerable extent its pathogenicity for man and dog.

By means of a comparable method the virus of yellow fever has been modified (1). By serial propagation in mouse brains its viscerotropic affinity is diminished. A highly virulent strain can by this means be readily converted into a relatively avirulent one which nevertheless acts as an efficient immunizing agent. This fact has been made use of in developing methods for immunizing man (2, 3).

However, by serial propagation in mouse brains, the inherent neurotropic affinity of yellow fever virus is markedly enhanced, not only for the mouse, but also for the monkey (4, 5), and probably for man. This increased neurotropism has rendered the virus potentially dangerous for human vaccination. Consequently a search was made to find methods for modifying yellow fever virus—methods which would not only reduce its viscerotropism but would also diminish, or at least not augment, its neurotropism. That this would be possible appeared from the evidence brought forward by the work of Lloyd, Theiler, and Ricci (6), which indicated that by prolonged cultivation in a tissue

culture medium the viscerotropic affinity of a highly virulent strain of yellow fever virus was markedly diminished, without at the same time producing a marked change in the neurotropic affinity. The virus cultivated in tissue culture was significantly less neurotropic than the virus then in use for human vaccination. This culture virus was consequently substituted (6) for the French neurotropic strain in the method of vaccination used by Sawyer, Kitchen, and Lloyd (2). The attenuation of the viscerotropic affinity produced *in vitro*, however, was not deemed sufficient to warrant the use of this virus without additional protection by the immune serum.

In the previous paper (7) we have presented evidence to show that by the prolonged cultivation in a medium containing minimal amounts of nervous tissue, both of the major affinities are greatly diminished. The virus obtained by propagation in this medium produced extremely mild reactions when inoculated subcutaneously into *rhesus* monkeys. Furthermore, the virus had lost the power of producing fatal encephalitis when injected intracerebrally into these animals. The ability to produce fatal encephalitis in monkeys was lost between the 89th and the 114th subcultures *in vitro*. A comparison of the neurotropic affinities for mice, using the virus obtained from the 114th and the 176th subcultures, showed that the virus from the latter subculture was considerably less neurotropic than that from the earlier. This attenuated virus acted in monkeys as an efficient immunizing agent to a subsequent subcutaneous inoculation of a highly virulent strain. The idea of using this virus without the simultaneous injection of immune serum was obvious. However, before introducing this virus for human vaccination additional experiments were undertaken.

Methods and Materials

The virus used in this study was the so called virus 17 D, which for a long period had been cultivated *in vitro* in a medium containing the tissues of chick embryos from which the head and spinal cord had been removed (7). The methods and materials used in cultivating the virus of yellow fever have been described in previous papers (6, 7). The virus is present in the tissue cultures in rather low concentration. In studying the immune response of monkeys to large amounts of virus, the requisite concentration of virus for inoculation purposes was obtained by utilizing brains of mice which had been infected with tissue culture material.

Vaccines for human use were prepared from infected whole chick embryos inoculated with culture virus by the technique of Elmendorf and Smith (8). This insures a high concentration of virus. Infected chick embryos were ground up in a mortar with normal human serum to make a 10 to 15 per cent suspension. After centrifugation this suspension was passed through a Seitz filter and the filtrate frozen and desiccated in tubes, each containing from 0.5 to 1.0 cc. Aerobic and anaerobic cultures were made on the filtrate to test for bacterial sterility. The potency of the vaccine was tested as routine by titration in mice by intracerebral inoculation.

EXPERIMENTAL

Response of Rhesus Monkeys to a Subcutaneous Inoculation of Various Amounts of Virus.—Two experiments were performed to study the response of *rhesus* monkeys to various amounts of virus.

In the first of these experiments eight monkeys were used. The virus was titrated in mice, and in Table I the estimated number of average lethal doses for mice which each monkey received are shown. The first two monkeys (Nos. 1 and 2) were inoculated with a suspension of infective mouse brains prepared from mice inoculated with material from the 216th subculture. The remaining six monkeys were inoculated with decimal dilutions of the supernatant fluid from the 217th subculture. In the blood of five no virus was demonstrated after inoculation. In three monkeys (Nos. 1, 2, and 4) the presence of virus in the blood was shown for a duration of 1, 2, and 3 days respectively. Two animals (Nos. 1 and 8) responded with a febrile reaction of 1 day's duration, in one animal 1 day after inoculation, and in the other 5 days. All eight animals lived, and seven were shown to have developed neutralizing antibodies in their sera 1 month after inoculation. There seemed to be no correlation between the titer of the serum antibodies and the size of the immunizing dose. One animal (No. 6) failed to become immunized for reasons not apparent, and when inoculated later with a somewhat larger dose of the same tissue culture virus it readily responded with the production of antibodies. The seven monkeys which showed a demonstrable antibody production were given a test dose of French neurotropic virus administered intracerebrally. This method of testing the immunity of a monkey is far more severe than that of inoculating an animal intraperitoneally with the highly virulent Asiatic virus, which is generally used. Four of the animals (Nos. 2, 3, 7, and 8) responded to this immunity test with a febrile reaction; two of these developed signs of encephalitis (Nos. 3 and 8), one of which (No. 8) died on the 7th day of yellow fever virus encephalitis. Three normal control monkeys inoculated intracerebrally with the same virus preparation died of encephalitis in 7 to 9 days. It may be significant that the two animals which showed signs of encephalitis following the test dose of neurotropic virus were those which also showed the least amount of neutralizing antibodies.

TABLE I
The Response of Rhesus Monkeys to a Subcutaneous Inoculation of Varying Amounts of Virus 17 D

Mon- key No.	Inoculum		Test for virus in circulating blood: mortality ratio in mice inoculated with serum*										Fever on days after inocula- tion	Results	Antibody titer 30 days after inoculation	Immunity test: French neurotropic virus intracerebrally		
	Sub- culture	Estimated No. of mouse M.L.D.	Route	Days after inoculation												Fever	Results	
				1	2	3	4	5	6	7	8	9						10
1	216	73,000,000	i.p.	3/7	0/5	0/4	0/6	0/6	0/6	0/6	0/6	0/4	0/6	5	Lived	1:19	—	Lived
2	"	30,000,000	i.v.	6/6	6/6	0/6	0/5	0/6	0/6	0/6	0/6	0/6	0/5	—	"	1:32	5, 6, 7, 8	"
3	217	13,000	s.c.	0/50	7/0	0/7	0/6	0/5	0/6	0/6	0/6	0/6	0/6	—	"	1:2	6, 7	Encephalitis, lived
4	"	1,300	"	0/50	0/6	0/6	0/6	2/5	2/5	5/6	0/6	0/6	0/6	—	"	1:134	—	Lived
5	"	130	"	0/7	0/6	0/6	0/5	0/6	0/6	0/6	0/5	0/5	0/6	—	"	1:5	—	"
6	"	13	"	0/6	0/5	0/7	0/6	0/6	0/6	0/6	0/6	0/6	0/6	—	"	Negative	Not tested	
7	"	1.3	"	0/50	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/50	0/6	—	"	1:37	5, 6, 7, 8	Lived
8	"	0.13	"	0/7	0/6	0/4	0/6	0/6	0/6	0/6	0/6	0/6	0/5	1	"	1:2	4, 5, 6	Died of enceph- alitis

i.p., intraperitoneally.

i.v., intravenously.

s.c., subcutaneously.

* The numerator represents the number of mice that succumbed to infection; the denominator, the number of mice used in the test.

The influence of the quantity of virus in the immunizing dose on the resulting immunity was tested in a second experiment essentially the same as the above.

Six monkeys were inoculated subcutaneously with 1.0 cc. of decimal dilutions of the supernatant fluid of the 215th subculture of the virus in chick embryo tissue. Titration of the virus by intracerebral inoculation of mice showed that the monkeys were inoculated with 0.2 to 20,000 minimum lethal doses for mice. Two animals (Nos. 14 and 16) had a febrile reaction, one (No. 14) of 1 day's duration on the 4th day, and the other (No. 16) of 3 days' duration commencing on the 5th day after inoculation. All the animals lived. The antibody titer

TABLE II

Antibody Response of Rhesus Monkeys Inoculated Subcutaneously with Varying Amounts of Virus

Monkey No.	Inoculum			Serum antibody titer				
	Sub-culture	Route of inoculation	No. of mouse M.L.D.	Before inoculation	Weeks after inoculation			
					4	6	8	12
9	227	s.c.	33,000	0	1:6	1:27	1:33	
10	"	"	33,000	0	1:5	1:33	1:47	
11	215	"	20,000	0	1:8	—*	—	1:4
12	"	"	2,000	0	1:4	—	—	1:3
13	"	"	200	0	1:2	—	—	1:240
14	"	"	20	0	1:2	—	—	1:28
15	"	"	2.0	0	1:2	—	—	1:4
16	"	"	0.2	0	1:8	—	—	1:26

* Not tested.

of sera obtained 1 and 3 months after immunization was determined. The results showed that the antibody titer of the sera obtained 3 months after vaccination was appreciably higher than that obtained after 1 month in four of the six animals. These results are shown in Table II. Included in this table are results of titrations on the sera of two additional monkeys (Nos. 9 and 10) obtained 4, 6, and 8 weeks after vaccination. These two monkeys had been inoculated subcutaneously with 1.0 cc. of a tenfold dilution of a virus preparation representing the 227th subculture. Some of this preparation was later used for human inoculation. As routine in immunizing animals, we have taken the serum antibody titer 1 month after vaccination as an index of the efficacy of the method of immunization. From the results on these animals it is obvious that an entirely erroneous conclusion may be obtained by using this arbitrary time interval, and

it is possible that had the antibody response of all our animals been determined over a longer period of time following inoculation, higher titers would have been found than those recorded throughout this and the previous paper (7).

From the results of these experiments undertaken to determine what influence the quantity of virus in the immunizing inoculation has on the resulting immunity, the conclusion seems warranted that even minimal quantities have the ability to produce an infection and consequently the production of antibodies and immunity. Both monkeys (No. 8, Table I, and No. 16, Table II), inoculated with only fractions of an average lethal dose for mice, developed antibodies. This result seems to suggest that the monkey is probably more susceptible to the virus than the mouse, or at least more uniformly susceptible. Some mice, of the so called Swiss strains used in our work, will become infected even when inoculated with fractions of an average lethal dose. This result is to be expected from the method of determining the average lethal dose, which by definition is the amount of virus which kills half the mice inoculated within a specified period of time.

Time of Appearance of Immunity in Monkeys Following Inoculation.

—The time after inoculation when immunity is developed can be determined by two methods. The first is the determination of the time after inoculation when demonstrable antibodies appear in the blood. Antibodies were shown to be present as early as 7 days after inoculation in several monkeys investigated. After 14 days all animals studied had demonstrable antibodies. The second method of determining the time of development of immunity is to inoculate monkeys with a virulent virus at various intervals after the immunizing inoculation. Table III shows the result of such an experiment.

The virus preparation used for the immunity test was a frozen and desiccated serum obtained from a monkey at the height of infection with the virulent Asibi strain. This preparation had been found to be virulent when tested on numerous occasions. All the monkeys used in this experiment were first inoculated subcutaneously with 1 cc. of a 1 in 10 dilution of the tissue culture vaccine. The first monkey in the series (No. 17) received the test dose of Asibi virus immediately after the vaccine. In the other monkeys the interval between the vaccine and the test dose was 1, 3, 5, 7, and 14 days. Two monkeys (Nos. 25 and 26) were inoculated with the test virus alone to serve as controls. Following the inocula-

tion of the Asibi virus, the monkeys were bled daily and the sera obtained injected intracerebrally into mice to determine the presence of virus in the circulation. Monkey 17, which was inoculated simultaneously with the vaccine and the Asibi virus, died of typical yellow fever 3 days after inoculation. Two monkeys (Nos. 18 and 19) which received the test virus 1 and 3 days after the vaccine died of yellow fever 4 and 6 days respectively after the test. Monkey 20, in which the interval between the immunizing and the test inoculation was 5 days,

TABLE III

The Response of Rhesus Monkeys to a Test Dose of Virulent Asibi Virus Injected at Various Time Intervals after Vaccination with Tissue Culture Virus 17 D

Mon- key No.	Time interval between vac- cination and immunity test with Asibi virus	Test for virus in circulating blood: mortality ratio in mice inoculated with monkey serum							Fever on days after inocu- lation	Results	Presence of antibodies in serum after vaccination with virus 17 D	
		Days after injection of Asibi virus									7 days	14 days
		1	2	3	4	5	6	7				
	days											
17	0	3/6	7/7	5/5					—	Died on 3rd day		
18	1	0/7	7/7	6/6					3	Died on 4th day		
19	3	0/6	6/6	5/5	7/7				4	Died on 6th day		
20	5	0/7	3/7	0/6	1/5	0/7	0/6	0/6	4, 6	Lived		
21	7	0/6	0/3	0/7	0/6	0/5	0/6		—	"	Negative	Positive
22	7	0/6	0/6	0/7	0/6	0/7	0/5		—	"	"	"
23	14	0/6	0/4	0/6	0/4	0/6	0/5		22	"	"	"
24	14	0/6	0/6	0/6	0/2	0/6	0/5		18	"	"	"
25	Unvaccinated control	1/4	8/8	8/8	0/5	0/6	0/6	0/5	3, 4, 5	"		
26	" "	4/6	5/6	8/8	5/6	3/6	0/6	0/6	—	"		

* Result inconclusive as three of six mice survived.

showed a febrile reaction but lived. In this animal minimal amounts of virus were demonstrated in the circulation. Four animals (Nos. 21, 22, 23, and 24), in which the intervals were 7 days and 14 days, all lived and showed no virus in their circulation during the first 6 days following the test inoculation. The fevers of 1 day's duration on the 18th and 22nd days following inoculation registered by two monkeys cannot be considered specific. Neutralization tests with the sera obtained from these four animals 1 and 2 weeks after the immunizing injection showed that at the latter period of time demonstrable antibodies were

present in all four animals. The two control monkeys (Nos. 25 and 26) inoculated with the test virus alone unexpectedly lived. Virus was demonstrated in the blood of both, in one animal (No. 25) for 3 days and in the other (No. 26) for 5 days.

In spite of the failure of the two control animals to die, the experiment is not without value. Using presence or absence of circulating virus as an index of immunity, the conclusion seems warranted that a substantial immunity is present 1 week after the immunizing inoculation and a partial immunity in 5 days.

A Comparison of the Pathogenicity of the French Neurotropic Virus and the 17 D Strain of Culture Virus for Experimental Animals.—The two strains of yellow fever virus which have been used to date for the vaccination of man are the French virus, modified by serial mouse brain passage, and the Asibi virus, modified by cultivation in whole mouse embryo.

Of these two, only the first has been used alone, without the simultaneous injection of immune serum (3, 9-11). The pathogenicity of the French neurotropic virus for experimental animals is known, and a certain amount of information is available concerning its pathogenicity for man. *Rhesus* monkeys inoculated intraspinally or intracerebrally with the French neurotropic virus invariably die of yellow fever virus encephalitis (4, 5). Following extraneural inoculation this virus produces an infection which manifests itself by the production of a febrile reaction in approximately 50 per cent of the animals. In Table IV are summarized the results obtained by inoculating twenty-one monkeys by various extraneural routes with varying amounts of French neurotropic virus. Virus in the circulation, lasting for a period of 2 to 6 days, was invariably present; and approximately 30 per cent of the animals died of encephalitis. By mouse brain passage the viscerotropic affinity of the virus has been considerably diminished, whereas the neurotropic affinity has been augmented. It is this enhancement of the neurotropic affinity which makes this virus potentially dangerous for human vaccination. That this fear is justified has been proven by the occurrence of severe involvement of the central nervous system following its use for human vaccination. These neural accidents have occurred both when the virus was administered alone (10, 12) as well as when administered with immune

TABLE IV
The Pathogenicity for Rhesus Monkeys of the French Neurotropic Virus When Injected by Extraneural Routes

Monkey No.	Characteristics of virus			Test for virus in circulating blood; mortality ratio in mice inoculated with monkey serum										Fever on days after inoculation	Results
	No. of mouse brain passages	Route of injection		Days after inoculation											
		Inoculum, mouse brain	g.m.	1	2	3	4	5	6	7	8	9	10		
27	117	0.005	s.c.											3, 6, 7, 10	Lived
28	"	0.005	"											—	"
29	119	0.000,001	"		0/5	0/6		0/6		2/6				Continuous 14th to 19th	Died on 24th day of encephalitis
30	"	0.000,001	"			6/6		6/6		0/6				6th to 9th	" " 11th " " "
31	"	0.000,001	"			0/6		0/6		6/6				—	" " 10th " " "
32	176	0.003	"			2/6		1/6		0/6				2, 3, 4, 6	Lived
33	"	0.003	"	3/6	4/4		0/6		0/6					6, 7	"
34	200	0.01	"	5/11	12/12	11/12	12/12	11/12	11/12	1/12				10	Died on 12th day, cause undetermined
35	"	0.01	i.p.	0/12	6/11	11/11	11/12	6/12	6/12	0/10				—	Lived
36	"	0.01	"	7/12	12/12	11/11	12/12	9/11	9/11	1/12				5, 6, 10	"
37	227	0.8	"	7/7	6/6	6/6		2/4	2/4	0/6				2, 3, 4	"
38	"	0.00001	"	0/6	0/6	4/6	5/6	3/3	3/3	5/6				9, 12, 13	"
39	"	0.5	"	6/6	5/5	6/6	6/6	6/6	6/6	0/6				4, 5, 6	Died on 9th day, cause undetermined
40	"	0.5	"	5/6	6/6	6/7	3/6	5/6	5/6	0/3				5	Lived
41	"	1.5	"	6/6	6/6	0/6	0/6		0/6	0/6				6	Died on 8th day of encephalitis
42	"	1.5	"	6/6	6/6		0/6		0/6	0/6				—	" " 18th " " "
43	293	0.01	Scanned							0/6				—	Lived
44	"	0.01	"	0/6		6/6		4/6	4/6	1/6				—	Died on 12th day of encephalitis
45	"	0.01	"			5/6		5/6	5/6	0/7				—	" " 12th " " "
46	"	0.01	s.c.	2/6		6/6		6/6	6/6	1/6				5, 6, 7	" " 10th " " colitis
47	"	0.01	"	5/5		6/6		3/6	3/6	0/6				—	" " 11th " " encephalitis

serum (13). Not only does the French neurotropic strain occasionally produce severe neural involvement, but the systemic reaction of man to the virus when administered without immune serum is sufficiently severe in a fair proportion of people to contraindicate its use.

The known pathogenic activities for experimental animals of the French neurotropic virus and virus 17 D are summarized in Table V. In all the points enumerated the French neurotropic virus is more pathogenic than the cultivated strain. It is a noteworthy fact, however, that though the French neurotropic virus is extremely pathogenic

TABLE V

A Comparison of the Pathogenicity of French Neurotropic Virus and Virus 17 D for Experimental Animals

Virus	Mice	Rhesus monkeys		Hedgehogs
	Average time of death after intracerebral inoculation	Intracerebral inoculation	Extraneural inoculation	Subcutaneous inoculation
French neurotropic	days 4-10	Fatal encephalitis	Fever in approximately 50 per cent of animals. Virus present in circulating blood for a period of 2-6 days. Fatal encephalitis in approximately 30 per cent of animals	Death from encephalitis with liver necrosis
Tissue culture virus 17 D	8-20	Non-fatal encephalitis	Occasional fever. Minimal amounts of virus in the circulation: No deaths	Animals survive

for the nervous systems of all the susceptible experimental animals, the number of times the central nervous system has become involved in man following the use of this virus for vaccination is relatively small. The danger of similar accidents occurring in man following the use of the strain cultivated in a medium poor in nervous tissue should accordingly be negligible. After 114 subcultures this virus had lost its power of producing fatal encephalitis in monkeys. That continued cultivation in chick embryo medium after the 114th subculture leads to a further loss of neurotropism is shown by the intracerebral inoculation of mice with later subcultures. From the results

of these animal experiments it is felt that the virus grown in chick embryo tissue for more than 200 subcultures should be safe for human vaccination.

Inoculation of Immune Persons with Tissue Culture Virus 17 D.—As the immune response in monkeys following vaccination with the virus grown for prolonged periods in chick embryo tissue indicated that this response was comparatively mild, it seemed desirable to test the response in immune persons before using this virus for human vaccination. In Table VI is shown the antibody production in four persons inoculated subcutaneously with a vaccine prepared from the 127th subculture of the virus grown in chick embryo tissue. One of

TABLE VI

Antibody Response in Immune Persons to a Subcutaneous Inoculation of Tissue Culture Virus 17 D

Immune persons inoculated	Inoculum			Serum antibody titer										
	Amount	Subculture	No. of mouse M.L.D.	Before inoculation	Weeks after inoculation									
					1	2	3	4	5	6	7	8	9	10
M. T.	1.0	227	330,000	1:32		1:90		1:125		1:110		1:80		1:96
H. S.	1.0	227	330,000	1:2	1:2	1:4	1:3	1:8		1:5		1:21	1:8	
T. F.	0.8	227	70,000	1:2	1:6	1:100	1:100	1:100						
R. L.	0.5	227	44,000	1:2	1:2	1:25	1:20	1:34						

them had an antibody titer of 1 in 32 before inoculation, as a result of an attack of yellow fever several years before. The three other persons had been vaccinated previously, but the antibodies had almost entirely disappeared from their sera. In all four there was a marked antibody response. Apart from a slight local reaction at the site of inoculation no signs or symptoms were noticed.

The antibody response in immune persons to a subcutaneous inoculation of virus grown for a prolonged time in chick embryo tissue differs markedly from the response produced by the French neurotropic virus and virus grown in mouse embryo tissue. Lloyd, Theiler, and Ricci (6) found that the antibody titer in immune human beings, inoculated with the two strains of virus mentioned above, rose

TABLE VII
Results of Vaccination of Non-Immune Persons with Tissue Culture Virus 17 D

Non-immune persons			Virus		Fever on days after inoculation	Highest temperature recorded	Serum antibody titer						
Laboratory number	Age	Sex	Sub-culture	No. of mouse M.L.D.			Before inoculation	1	2	3	4	6	8
	yr.				day	°C.							
164	45	M	227	50,000	7th	37.2	Neg.	Neg.	1:2	1:8	1:10	1:10	1:32
166	42	M	"	50,000	1st, 3rd	37.2	"	"	1:2	1:6	1:10	1:3	1:4
168	40	F	"	50,000	—		"				Pos.*		
169	42	M	"	50,000	7th	37.3	"		Pos.*		"		
171	35	F	"	50,000	5th, 6th	37.3, 37.2	"						
172	12	F	229	3,000,000	—		"		Pos.*				
173	35	F	"	3,000,000	—		"		"				
175	38	F	"	3,000,000	9th, 10th	37.4, 37.2	"		"				

* Antibody titer not determined.

rapidly to reach a peak on about the 14th day and then rapidly diminished, so that the titer 4 weeks after inoculation tended to approximate its initial level. The antibody response of immune persons to the virus grown in chick embryo tissue is much slower. From the limited number of observations recorded in this paper, it would appear that the antibody titer rises gradually, the height of the rise not being reached until the 4th to the 8th week, when a slow decline sets in. Even after 10 weeks the antibody titer has not fallen to its initial level.

Inoculation of Non-Immune Persons with Tissue Culture Virus 17 D.—To date eight normal persons have been vaccinated with the 17 D culture virus. The vaccines used were prepared from the 227th and the 229th subcultures. The relevant observations on these persons are shown in Table VII. The reactions at the site of inoculation were minimal. Five persons had a febrile reaction, which in four of them occurred from the 5th to the 7th days. The highest temperature recorded was 37.4°C. The febrile reactions were accompanied as a rule by slight headache and backache, which were not severe enough to prevent the subject from following his normal occupation.

Investigations on the appearance of antibodies showed that, 2 weeks after vaccination, demonstrable antibodies were present in the serum of all the six persons studied. The sera from three showed no protective antibodies 1 week after vaccination. The serum antibody titer has been studied in only two subjects. In both the antibody titer was determined at intervals after vaccination. The results tend to show that the antibody titer produced is very low. No information is available as to the duration of the immunity produced by vaccination.

The results obtained in the small number of persons vaccinated with the tissue culture virus are sufficiently encouraging to warrant a more extensive trial of the method.

SUMMARY

The response of *rhesus* monkeys to a subcutaneous inoculation with varying amounts of virus modified by prolonged cultivation *in vitro* has been studied. The tissue components of the medium consisted of chick embryo tissue containing minimal amounts of nervous tissue.

The immunity produced in monkeys, as measured by the antibody titer developed, has no relation to the amount of virus inoculated.

Monkeys inoculated subcutaneously with the tissue culture virus are rendered immune to a subsequent injection of a highly virulent yellow fever virus. This resistance is already present 7 days after vaccination.

The subcutaneous inoculation of the culture virus into immune persons leads to a substantial increase of the serum antibody titer.

The results of vaccinating eight normal persons with culture virus are presented. The reactions were minimal. The highest temperature recorded following vaccination was 37.4°C.

The sera taken from the eight vaccinated persons 2 to 4 weeks after inoculation with the tissue culture virus showed the presence of yellow fever antibodies.

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THE ADAPTATION OF UNMODIFIED STRAINS OF YELLOW FEVER VIRUS TO CULTIVATION IN VITRO

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Using a technique similar to that devised by Rivers (1) for the cultivation of vaccinia virus, Haagen and Theiler (2) reported successful cultivation of a strain of yellow fever virus which had been greatly modified through many mouse passages by intracerebral inoculation. The medium consisted of chick embryo tissue and Tyrode solution containing 10 per cent of normal monkey serum. However, efforts to cultivate unmodified strains of yellow fever virus failed (3). After many attempts, Lloyd, Theiler, and Ricci (4) finally succeeded in establishing the unmodified Asibi strain in tissue cultures in which mouse embryo was used as the tissue component of the medium in place of chick embryo tissues. This strain of virus has now been under continuous cultivation in this laboratory for over 3 years.

The Asibi strain of yellow fever virus is the most virulent both in viscerotropic and in neurotropic properties so far isolated. Through continued cultivation *in vitro*, it has lost much of its pathogenicity (4, 5) and is used at present for human vaccination against yellow fever (6). However, as practically nothing is known regarding the permanency of the changes that take place in the virulence of the virus during prolonged *in vitro* cultivation, it seemed desirable to adapt strains of less initial pathogenicity to cultivation in tissue cultures. Many of the strains of virus recently isolated from human cases of yellow fever are of relatively low virulence and only moderately pathogenic for *rhesus* monkeys. It was considered probable that these strains would lose their virulence in tissue cultures within a shorter time and with less danger of reversibility than the highly virulent Asibi strain.

Method

The technique, in general, was briefly as follows:

All cultures were made in 50 cc. Erlenmeyer flasks closed with cork stoppers wrapped with lead foil. The fluid component of the tissue culture medium consisted of 10 per cent normal monkey serum in Tyrode solution. 4 cc. of this mixture were used as routine in each flask. To this were added 2 large drops of tissues, which had been minced with a tissue crusher or chopped finely with scissors. In a few experiments the plasma clot technique was tried in place of liquid medium. The source of virus used to inoculate the cultures consisted of either serum from an infected monkey or a Seitz filtrate of a 10 per cent infected mouse-brain suspension. With a few exceptions 1.0 cc. of the original virus-containing material was generally used to inoculate each flask and a similar amount of an old culture was added to fresh medium when subsequent transfers were made. Subcultures were usually made at intervals of 3 to 7 days. The cultures were incubated at 37°C.

EXPERIMENTAL

This technique which was so successfully applied to the cultivation of the Asibi strain was repeatedly tried with four additional unmodified strains of virus. Twenty-nine unsuccessful attempts to adapt the F. W. strain were made. In only nine experiments could virus be demonstrated in the primary culture after 3 to 7 days incubation. In one instance virus was present in the second subculture. At least fourteen similar attempts were made with three other strains of virus. None of the experiments gave promise of success.

As all these efforts to cultivate new strains of virus had failed, it was decided to approach the problem from a different angle. One possibility that suggested itself was the use of tissues of a susceptible animal such as the *rhesus* monkey. In order to determine to which particular tissue of the monkey the virus had greatest predilection, the following experiments were carried out.

Experiment 1.—A monkey was given intraperitoneally 3 cc. of serum from another monkey infected with the F. W. strain of yellow fever virus. On the 3rd day after inoculation the monkey had fever. On the 4th day it was exsanguinated under anesthesia. The blood was allowed to clot and the serum removed. On intracerebral test in mice the serum was found to have a moderate amount of virus, and proved infective for mice when inoculated intracerebrally with a dilution of 1 in 1,000. The spleen, ovaries, a piece of the liver, and both femurs were removed

aseptically from the monkey. The bone marrow was then extracted from the femurs under sterile precautions. A series of cultures was then made using the infected monkey tissues as the source of virus, as well as the tissue component in the serum-Tyrode medium. All the cultures were incubated at 37°C. for 7 days, after which their infectivity was tested in mice by intracerebral inoculation. The results showed that among the cultures with various tissues those which contained bone marrow seemed most favorable for virus preservation. This experiment was repeated several times, using F. W. as well as the French strain of virus, and similar results were obtained. Although the virus seemed to have a greater predilection for the bone marrow, when attempts were made to maintain it in a medium containing bone marrow tissues, it was invariably lost after the second or third subculture.

Experiment 2.—It occurred to us that monkey tissues taken shortly after the animal had been infected with virus might give a more favorable cellular medium for virus multiplication. Thus the use of cells rendered necrotic by the infection and the possible presence of antibodies in the animal tissues on the 3rd or 4th day of infection might be avoided. Accordingly, three monkeys were each given a large dose of F. W. strain of virus intravenously, and were killed by exsanguination under anesthesia at 5, 24, and 48 hours, respectively, after inoculation. The bone marrow, spleen, testicle, and an adrenal were removed from each monkey and culture made in exactly the same manner as in the preceding experiment.

The virus content of all the tissues used for culture purposes was determined by the intracerebral inoculation of mice. With the exception of the blood, which showed only a trace, no virus was demonstrated in any of the organs of the monkey killed 5 hours after inoculation. The monkey killed 24 hours after infection had demonstrable virus in the blood and liver but in none of the organs used for culture purposes. The third animal of the series killed 48 hours after infection with the F. W. virus showed considerable amounts of virus in the bone marrow and only small quantities in the spleen and testicle.

After 4 to 7 days' incubation, subcultures were made by using as an inoculum a suspension of the ground up tissue fragments of the original culture. The tissue used for preparing the subcultures was obtained from a normal monkey. At the time when subcultures were made, mice were inoculated intracerebrally with the material used for making transfers. The results of these inoculations showed virus to be present in only three of the cultures. None of the cultures prepared from the tissues of the monkey killed 5 hours after infection showed virus. In the cultures made from the 24 hour monkey, only those having adrenal gland showed virus. Cultures prepared from spleen and bone marrow of the 48 hour monkey showed a small amount of virus. Infectivity tests with material from the first subcultures

CULTIVATION OF YELLOW FEVER VIRUS

demonstrated virus to be present only in the cultures containing adrenal gland tissue, in the series initiated from the monkey killed 24 hours after infection.

Infection of Mouse Embryos in Utero with Yellow Fever Virus.—The successful cultivation by Lloyd, Theiler, and Ricci (4) of the unmodified Asibi virus in a medium containing mouse embryo tissues, suggested that in the mouse embryo there must be some groups of cells which serve as a favorable medium for the propagation of the virus. On the other hand, the fact that the concentration of the virus in mouse embryo tissue culture was never very high seemed to indicate that the proportion of such cells was relatively small in the whole embryo. With a view to determining to which particular organ the virus has the greatest predilection, embryos were infected *in utero*, and later the relative virus content of the various organs determined as follows:

The technique used for inoculation of the mouse embryos *in utero* was similar to that described by Woolpert (7) in his study of bacterial infections in mammalian embryos. The Swiss mice about the 13th day of pregnancy were used. The mouse was anesthetized with ether and tied on a board so as to expose the abdomen. An incision was made in the skin over one side of the abdomen and by careful dissection an area of the peritoneum about 1 square cm. was exposed. The embryos could be seen through the peritoneum and by some manipulation all the embryos in one uterine horn could be inoculated. Using a tuberculin syringe and a 26 gauge needle, each embryo was inoculated with 0.02 cc. of the virus-containing material.

These experiments were carried out with three different strains of the virus. At the end of 4 days, virus could be recovered from the inoculated embryos in considerable amounts as demonstrated by the intracerebral mouse test of a suspension of embryo tissue. After inoculation, however, the pregnant mice aborted so often that the continuous passage of virus from embryo to embryo was found to be very difficult. This study was consequently limited to the determination of the distribution of virus in the infected embryos, in order to learn which tissue was the most suitable for virus multiplication.

Experiment 1.—The embryos contained in the right uterine horn of three Swiss mice were inoculated on Feb. 20, 1936, with the serum of a monkey infected with the Asibi strain of virus. 4 days later one mouse aborted. From the other

two mice a total of seven embryos were removed aseptically. They were carefully dissected and various organs were removed separately, which included the brain, liver, kidney, a large proportion of the skin, the abdominal and thoracic viscera, and legs and tail. The homologous organs of the seven embryos were pooled and weighed. They were then ground finely in a sterile mortar and suspended in a diluent consisting of 10 per cent normal monkey serum in saline. The infectivity of each organ pool was tested for virus by intracerebral injection of 1:10 and 1:100 dilutions into mice.

The results indicated that with one exception all suspensions were infective in a dilution of 1 in 100, the exception being the suspension made of the abdominal and thoracic viscera of the seven embryos, which proved to be non-infective.

As no satisfactory conclusion could be drawn from this experiment with regard to the relative concentration of virus in the different mouse embryo tissues, the same procedure was repeated, using higher dilutions of the infected tissues.

Experiment 2.—The experiment just described was repeated with another group of embryos infected *in utero*. The Asibi strain of yellow fever virus was again used and the embryos were removed for titration at the end of a 4 day period after inoculation. In this experiment the decimal serial dilutions of the pooled organ suspensions were tested for presence of virus by intracerebral inoculation in mice up to 1 in 100,000.

The results are shown in Table I. It will be seen from the results that while the titration of tissues showed a wide variation in the distribution of the virus throughout the body, the mouse embryo brain contained a higher concentration than any other organ, as no endpoint had been reached in a dilution of 10^{-5} . The thoracic and abdominal viscera which gave entirely negative results in the preceding experiment contained relatively small amounts of virus in this test.

The Cultivation of Unmodified Strains of Virus in Vitro in the Presence of Mouse Embryo Brain Tissues.—In accordance with the information obtained from the above experiment, attempts were made to cultivate unmodified strains of virus in a medium in which the tissue component consisted of minced mouse embryo brains. The brains were removed from 2 weeks old embryos under strict sterile precautions, and cultures prepared in the usual manner. Attempts to cultivate unmodified strains of virus in this medium proved highly successful.

CULTIVATION OF YELLOW FEVER VIRUS

Seven different strains of yellow fever virus were tried. They ranged in pathogenicity from the highly virulent French strain to newly isolated viruses from human cases of the so called jungle yellow

TABLE I
Titration of the Virus Content of Various Organs of Mouse Embryos Infected in Utero

Mouse embryo tissue tested	Dilutions of tissue tested	Result of intracerebral test for virus
Brain	10^{-1}	
	10^{-2}	5/6*
	10^{-4}	6/6
	10^{-5}	6/6
Liver	10^{-1}	6/6
	10^{-2}	7/7
	10^{-4}	3/6
	10^{-5}	0/7
Skin	10^{-1}	0/6
	10^{-2}	
	10^{-4}	5/5
	10^{-5}	5/6
Placentae	10^{-1}	4/6
	10^{-2}	2/6
	10^{-4}	6/6
	10^{-5}	6/6
Legs and tails	10^{-1}	0/5
	10^{-2}	0/6
	10^{-4}	
	10^{-5}	6/6
Other viscera	10^{-1}	6/6
	10^{-2}	5/6
	10^{-4}	2/6
	10^{-5}	
	10^{-1}	5/5
	10^{-2}	3/6
	10^{-4}	0/6
	10^{-5}	0/6

* The numerator represents the number of mice that succumbed to infection; the denominator, the number of mice used in the test.

fever which are low in neurotropic and viscerotropic properties. Of these, five were successfully adapted to tissue culture on the first attempt. Suspensions of infected mouse brains were used as a source

of virus to initiate the strains in the mouse embryo brain tissue cultures, and subcultures were made at 3 to 5 day intervals. In comparison with our former experience this was a striking success.

In our tissue culture work with yellow fever virus, the supernatant fluid of the centrifuged culture is tested as a routine procedure in mice for presence of virus by intracerebral inoculation each time a subculture is made. It was soon noted that the time required for encephalitis to develop in the mice used for these tests became shorter and shorter as the number of subcultures progressed, indicating an increase of neurotropism due to multiplication in the embryo brain tissue. As we wished to avoid an increase in neurotropism of the newly adapted strains by cultivating them too long in a medium rich in nerve tissue, an effort was made to replace the brain tissue with that of whole mouse embryo as early as possible during the *in vitro* cultivation. This substitution was unsuccessful in four different strains of virus when attempts were made at the fifth and sixth subcultures. When the virus strains had grown from twenty to twenty-five subcultures in mouse embryo brain, however, they were adapted to the whole mouse embryo with greater facility.

As the greatest attenuation of the Asibi virus had occurred in a medium, the tissue component of which was minced chick embryo tissue containing minimal amounts of nervous tissue (5), attempts were made to cultivate these new strains in similar media. The first attempts were unsuccessful. After longer periods of cultivation, however, it was found possible to maintain the virus in media, the tissue components of which were prepared from chick embryos from which the brain and spinal cord had been cut away before mincing. In a similar manner successful cultivation was obtained in mouse embryo tissue containing minimal amounts of nervous tissue.

SUMMARY

1. In a search for suitable tissues for the cultivation of yellow fever virus *in vitro*, mouse embryos were inoculated with this virus *in utero*. A titration for virus content of the various organs of the embryos indicated that the virus was present in the brain in greatest concentration.

2. Unmodified strains of yellow fever virus were readily adapted to

CULTIVATION OF YELLOW FEVER VIRUS

cultivation *in vitro* in a medium consisting of minced mouse embryo brain tissue and Tyrode solution containing 10 per cent normal monkey serum.

3. After a continued cultivation in mouse embryo brain tissue cultures for twenty to twenty-five subcultures, these strains were readily adapted to cultivation in whole mouse embryo tissue medium.

4. There is evidence to indicate that a prolonged cultivation of the virus in mouse embryo brain medium increases its neurotropic properties.

5. Attempts to employ monkey tissues for *in vitro* cultivation of yellow fever virus gave entirely negative results.

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TOXEMIA OF PREGNANCY IN THE RABBIT*

I. CLINICAL MANIFESTATIONS AND PATHOLOGY†

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PLATES 24 TO 26

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Toxic affections associated with pregnancy have been reported in several species of animals, notably the sheep (2) and guinea pig (3); it is also common knowledge among breeders that overfat animals of all kinds are apt to die during gestation or parturition, but the exact nature and pathogenesis of these disorders is not known. A highly fatal affection associated with pregnancy also occurs in the rabbit. This condition is of interest, not only as a disease of the rabbit, but also because it bears a close analogy to the toxemias of pregnancy in man and may provide a means for an experimental approach to problems connected with human toxemia.

Sporadic cases of this affection have been under observation in the laboratory breeding colony for several years. In the fall of 1935, the breeding colony was moved to Princeton, and with the resumption of breeding operations there was an unprecedented outbreak of fatal cases of toxemia. The present report is based on the study of material provided by this outbreak supplemented by data obtained from previous observations.

It should be made clear at the outset that, strictly speaking, the disease is not limited to pregnant rabbits, but occurs post partum and in pseudopregnant females. It is also evident that all cases of toxemia are not identical clinically or pathologically, but for the present no

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† The author wishes to express his appreciation to Miss Marion Orcutt who performed the chemical analyses associated with the clinical study of the disorder.

attempt will be made to classify cases except on the basis of apparent severity of the disease.

The object of the present paper is to report the results of clinical and pathological investigations, including the clinical history and symptomatology of the disease, blood chemistry and post mortem findings. Investigations bearing on the etiology of the disease will be presented in a subsequent paper.

Materials and Methods

The colony in which the disease occurs is maintained in active breeding service by this laboratory for the study of constitutional problems. The pedigrees and life histories of all animals are known, and the characteristics of the stock have been the subject of extensive investigation.

During the period under consideration the population was composed of fourteen pure breeds, including the Belgian, Beveren, Chinchilla, Dutch, English, Havana, Himalayan, Lilac, Polish, Rex, Sable and Silver Marten, Siamese Sable, French Silver and Tan breeds, and numerous hybrid lines. In November, 1935, the adult stock numbered 350 males and 650 females. Some of these animals were normal in all respects investigated; others showed minor abnormalities, and still others were heterozygous for lethal variations, but most of the population were known to be transmitters of some abnormality.

The colony is housed indoors in individual cages. The diet consists of hay, oats and a standard commercial ration which has been in use for several years. Frequent routine feedings are made and at all times the animals have access to a free supply of food and water. Previous to November, 1935, the colony was housed at The Rockefeller Institute in New York, but in the present situation in Princeton the only immediate changes in living conditions are larger quarters, more commodious cages and a new water supply.

The material for the present report is based on cases of toxemia which occurred between November, 1935, and December, 1936. During this period there were 72 fatal cases of the disorder. Diagnosis of non-fatal cases cannot always be made on a basis of symptomatology, and as facilities for blood analysis were limited and pathological examination of all suspected cases was impractical, the incidence could not be accurately determined. Clinical diagnosis was substantiated by chemical study of four cases that terminated in recovery, but the incidence of non-fatal cases was undoubtedly much greater, and the evidence at hand indicates that many instances of ante partum and post partum morbidity are mild cases of toxemia.

The facilities available did not allow constant examination of the colony, and because of the rapid course of the disease, the period of clinical observation was frequently limited to the terminal stages of the disease or affected animals were found dead. In a number of instances, however, the clinical course was followed from apparent health to complete prostration and a full history was obtained.

Complete autopsies were performed in all fatal cases and blood and various organs were cultured for bacteriological study. Pathological and bacteriological examinations were also made in two non-fatal instances of the disorder. Tissues for microscopic examination were fixed in Helly's and Petrunkevitch's (4) solutions and stained with hematoxylin and eosin. Pituitary glands were serially sectioned and contiguous sections stained with Mann's methylene blue and eosin or copper hematoxylin.

Blood studies included the determination of sugar, non-protein nitrogen, urea nitrogen, creatinine, uric acid, sodium chloride, calcium, inorganic phosphate, fat, cholesterol, acetone bodies, total serum proteins and albumin. The methods used in these determinations and the technical procedures followed were those recommended in a standard laboratory manual (5). No attempt was made to control the dietary status of animals at the time blood was obtained.

Chemical blood analyses have been made in twelve fatal and four non-fatal cases of toxemia. In addition, the blood of a large number of healthy females has been examined in order to determine normal values and the early or preclinical changes in toxemia. Thirty resting and six pregnant females of this series have continued in health to the present time and for comparative purposes are considered in subsequent paragraphs as a normal control group. Six other animals subsequently died of toxemia at periods ranging from 119 to 2 days after chemical blood examination, while five others showed abnormal values during apparent health, but to date have shown no clinical evidence of toxemia.

The clinical and pathological changes characteristic of the disorder suggested a toxic origin, and the possibility of food or water poisoning was examined both by feeding various constituents of the diet in excessive amounts to normal rabbits and by adding a metallic poison to the food.

Clinical Course

The symptomatology and clinical course of toxemia of pregnancy in the rabbit are variable. The disease may occur in a comparatively mild form followed by recovery, or pursue a rapid course to a fatal termination. The disorder in mild cases may escape clinical observation or be classified as a minor transitory disturbance, and its true nature not recognized until pathological examination following death from a subsequent attack or from unrelated causes shows healed lesions of toxemia. In typical cases, on the other hand, the disorder is obvious and presents a definite clinical picture.

Frequently, the disease sets in abruptly and from the beginning the manifestations are those of a sudden and severe intoxication. In other instances, the onset of severe manifestations is preceded by general signs of malaise which may be observed for 3 or 4 hours and gradually become more pronounced.

The signs of acidosis predominate the attack. Air hunger and dyspnea are apparent and acetone breath may be detected on close observation. Thirst is an early manifestation in some cases and may be extreme; occasional animals are found dead with their heads immersed in the water container. In other instances, however, the water intake is markedly diminished. Total suppression of urine is the rule in all cases, and in no instance has sufficient urine been found post mortem to permit a quantitative analysis. There is a loss of normal vigor and activity and the animals sit hunched in a corner of the cage with roughened coats and dull, lustreless eyes. They respond sluggishly to ordinary stimuli and the gait is slow and incoordinate when movement is forced. The ears are cold and the flow of blood in the marginal veins may be stopped with slight pressure. Convulsions occur in some cases and as a rule, are of the tonic type but may be clonic in character. Other animals remain lethargic, and generally, in all cases a comatose stage with relaxed sphincters, dilated pupils and widespread muscular asthenia precedes death. Cyanosis and marked respiratory distress with the appearance of a serosanguineous discharge from the nares are often terminal manifestations.

The period of obvious illness with conspicuous toxic signs in such cases is remarkably brief and rarely exceeds a few hours in duration. Frequently, the condition of a doe has been observed to change from apparent excellent health to complete prostration in the course of a half hour, and in only one animal under treatment has the clinical course lasted longer than 1 day. The condition of internal organs at autopsy indicates, however, that the course of disease may be of longer duration and that the disorder may be well advanced before clinical signs become apparent.

In non-fatal cases the manifestations of toxemia are mild and usually not specific, and clinical diagnosis can rarely be made on the symptomatology, but rests rather on the occurrence of a protracted period of illness following abortion or desertion of a litter early in the puerperium. Occasionally, rapid respiration, dilated pupils, or a slight cyanotic tinge about the lips and nares may be observed, but generally, the manifestations are no more than those of slight disorder such as are present in a number of minor disturbances. Convulsions do not occur and acetone breath has not been detected. General morbidity with loss of appetite may persist for a week or more during which there is some loss of weight due largely to muscular wasting but without any noticeable depletion in fat depots. Fluid intake and urine excretion are markedly diminished. Recovery is gradual and leaves no outward sign of disability.

There is pathological and chemical evidence that mild cases may occur without symptoms, and while breeding records frequently show the past occurrence of abortion or desertion which may have been associated with the attack, in other instances there is no indication of reproductive abnormality in the history of the animal. Fatal asymptomatic cases also occur in which no outward sign suggests the condition

of the animal, and sudden death in the midst of nest building or other physiological activity is the first indication of disturbance.

In general, therefore, one may recognize three types of disease. First, a typical toxemia with characteristic manifestations which usually terminates in death within a few hours after the signs are first noted; second, a milder disorder which presents less characteristic signs but persists for a week or more and is followed by recovery; third, an asymptomatic affection which usually escapes recognition at the time but, in rare instances, may lead to sudden death.

Relation to Pregnancy

The disease is predominantly a disorder of pregnancy, but typical cases also occur post partum and in resting animals. Of the 72 fatal cases that occurred between November 1, 1935, and December 1, 1936, 43 or 59.3 per cent were in pregnant does, 15 or 20.8 per cent in post partum and 14 or 19.4 per cent in resting females.

There were 66 fatal cases in multiparous females, an incidence of 10.3 per cent, and only 6 cases, or an incidence of 2.6 per cent, in primiparae.

A more detailed account of the incidence in relation to pregnancy will be presented in a subsequent paper.

Blood Chemistry

The results obtained by chemical analysis of the blood of animals with toxemia were controlled in two ways: first, by comparison with values obtained in resting females of similar genetic origin and second, by a comparison with values obtained from pregnant females of the same genetic groups.

Resting Controls.—The values obtained for various constituents of the blood of thirty healthy resting females are presented in Table I. These results are divided into six groups, each of which is composed of animals of like genetic constitution but differing in some respect from those of other groups. While a consideration of the significance of group differences is beyond the scope of this paper, it may be mentioned that the mean values for these groups do show significant differences in one or more respects. In like manner, the values obtained for animals with toxemia are variable, but in all instances the

TABLE I
Values for Blood Constituents of Apparently Healthy Resting Female Rabbits

Group	Whole blood						Plasma		Serum					Ratios		
	Sugar	Non-protein nitrogen	Urea nitrogen	Creatinine	Uric acid	Sodium chloride	Cholesterol	Fat	Calcium	Inorganic phosphate	Total protein	Albumin	Globulin	UN/NPN	P/Ca	A/G
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	gm. per cent	gm. per cent	gm. per cent			
I	150	27.96	9.03	1.21		472			13.48	3.07	4.34	2.61	1.73	0.32	0.22	1.5
	142.8	26.50	13.47	1.49		466			14.25		5.97	3.12	2.85	0.51		1.1
	167.6	40.89	16.42	1.17		456			14.08	6.41	4.79	2.94	1.85	0.40	0.45	1.6
II	145.6	32.47	12.78	1.51		442	116.8		13.13	6.84	5.62	3.58	2.04	0.39	0.52	1.7
	152.2	37.72	13.63	1.47		419	107.2		15.19	7.68	6.03	4.04	1.99	0.36	0.50	1.9
	148.6	41.64	14.33	1.52	0.91	478	95.2		13.81	6.46	5.86	3.81	2.05	0.34	0.46	1.8
	145.6	38.10	13.72	1.53	0.81	495	105.4		14.11	5.93	6.34	4.14	2.20	0.36	0.42	1.8
	152.2	35.43	13.15	1.53	0.78	479	95.2		12.99	6.12	5.28	3.07	2.21	0.37	0.47	1.4
	136.3	32.13	17.43	1.51	0.64	469	105.3		13.38	5.51	5.16	3.13	2.03	0.54	0.41	1.5
	134.6	30.60	10.56	1.67	0.63	434	102		13.91	4.78	5.02	3.21	1.81	0.34	0.34	1.7
	150.5	39.45	16.06	1.81	1.00	453	106.9		13.32	4.00	5.34	3.35	1.99	0.40	0.30	1.7
	142.8	34.86	13.89	1.68	0.80	442	106.4		13.77	4.59	6.21	3.25	2.96	0.39	0.33	1.1
	145.7	35.70	14.07	1.53	0.75	449	108.6		14.74	4.11	5.92	3.28	2.64	0.39	0.27	1.2
III	126	36.9	16.13	1.40		445	123	842.7	15.48	3.75	5.44	3.46	1.98	0.43	0.24	1.7
	151.6	28.3	14.87	1.33		446	129.4	1183		3.84	5.25	3.03	2.22	0.52		1.4
	153		11.37	1.51	0.67	485	112.8	968.2	13.72	5.71	5.39	3.09	2.30		0.41	1.3
	124	35.1	12.09	1.46	1.11	429	137.6	571.6	14.70	5.89	5.18	3.01	2.17	0.34	0.40	1.4
	130.3	40.5	12.50	1.44		475	132.2	517.9	13.58	6.37	5.53	3.43	2.10	0.30	0.46	1.6
	127	40.9	14.07	1.42		475	111.2	430.3	12.80	6.20	5.62	3.14	2.48	0.34	0.48	1.3
	167.6	37.5	15.51	1.52	0.68	458	124	502	13.58	7.38	5.00	3.16	1.84	0.41	0.54	1.7
	187.6	38.4	14.52	1.45	0.67	490	126.6	860.6	12.99	6.28	5.03	3.43	1.60	0.37	0.48	2.0
	138.8	35.4	13.23	1.52	0.55	442	115.8	403.3	14.35	5.86	5.40	3.50	1.90	0.37	0.40	1.8
	140.1	38.7	16.54	1.43	0.58	459	125	143.4	15.52	4.27	5.92	3.58	2.34	0.42	0.26	1.5

IV	112	32.85	16.18	1.31	470	113.2 117.2	15.03	4.68	5.71	3.78	1.92	0.49	0.31	2.0
	94.3	40.8	19.9	1.34			15.44	3.60	6.65	3.81	2.84	0.49	0.23	1.3
	137.6	41.66	19.72	1.56			14.50	3.65	5.57	3.63	1.94	0.47	0.25	1.8
	124		17.56	1.49			14.70	3.78	5.00	3.30	1.70		0.25	1.9
V	142.8	32.13	17.85	1.50	445		15.61	4.55	5.92	3.23	2.69	0.56	0.29	1.2
	148.5	40	20.70	1.61	444		18.83	3.39	4.60	3.28	1.32	0.52	0.18	2.5
VI	138.3	31.89	11.32	1.54	465		15.77	3.78	5.10	3.10	2.00	0.52	0.24	1.6

deviation from normal as represented by control values is much greater than the difference between the various healthy groups.

Pregnant Controls.—With the view of determining the chemical changes that normally occur in pregnancy, the blood of six healthy females was studied and the results are shown in Table II. The determinations are not sufficiently numerous to allow a statistical comparison, but the results obtained indicate that the variations in blood chemistry accompanying pregnancy are very slight.

TABLE II
Values for Blood Constituents of Apparently Healthy Pregnant Rabbits

Group	Whole blood						Serum					Ratios		
	Sugar	Non-protein nitrogen	Urea nitrogen	Creatinine	Sodium chloride	Cholesterol	Calcium	Inorganic phosphate	Total protein	Albumin	Globulin	UN NPN	P/Ca	A/G
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	gm. per cent	gm. per cent	gm. per cent			
I	159.5	34.50	18.9	1.17	430		14.84	4.88	4.35	3.17	1.18	0.55	0.32	2.7
	118.1	41.64	15.3	1.14	470		15.35	6.08	5.07	2.92	2.15	0.37	0.39	1.4
IV	147	38.43	14.89	1.19	465	111.2	16.29					0.39		
	131.5	26.94	15.73	1.26	465		15.81		4.26	2.83	1.43	0.58		2.0
	125	30.18	15.84	1.15	462		16.39		5.85	3.40	2.45	0.53		1.4
VI	127	41.36	20.82	1.45	470		15.15	4.14	4.84	3.13	1.71	0.34	0.21	1.8

Toxemia

Chemical blood studies have been made both in fatal cases of toxemia and in cases with comparatively mild symptoms which terminated in recovery. Moreover, in a general survey of the colony, analyses were made on animals which later died of toxemia, and in addition abnormal values suggestive of toxemia were found in a number of apparently healthy rabbits.

Fatal Cases of Toxemia.—Chemical blood values determined in twelve fatal cases of toxemia are shown in Table III. A notable feature of the disorder is the extreme difficulty of obtaining blood from the marginal ear vein, and a number of animals died during heart

TABLE III
Values for Blood Constituents in Advanced Stages of Fetal Toxemia of Pregnancy

Rabbit No.	Whole blood							Plasma	Serum						Ratios		
	Sugar	Non-protein nitrogen	Urea nitrogen	Creatinine	Uric acid	Sodium chloride	Cholesterol		Acetone	Calcium	Inorganic phosphate	Total protein	Albumin	Globulin	UN NPN	P/Ca	A/G
1	35.4	60.8	29.1	4.34							7.40	3.04	4.36	0.47		0.69	
2	89.3					460			13.92	9.37	5.90	2.82	3.08	0.83	0.67	0.91	
3	95.5	65.7	54.8	4.58							4.27	1.82	2.45			0.74	
4							125	++		7.80	5.38	2.87	2.51	0.47		1.14	
5	47.1	64.1	30.2	4.76													
6	167.6	107.1	54.9	2.98			106.4	++	9.97	8.84	3.20	1.27	1.93	0.51	0.88	0.65	
7	155.2	64.3	37.2	4.19			282.4	++	9.70	13.27	4.08	2.03	2.05	0.57	1.36	0.99	
8	146.4	62.5	27.6	5.59			93.6	+		14.42	3.90	2.20	1.70	0.44		1.29	
9	309.2	140.1	107.1	8.3	1.23	564	306	++	8.82	11.71	6.13	3.09		0.76	1.32	1.0	
10	425.2	191	159.5	12.12			395		11.40	8.89	5.54	2.22	3.32	0.83	0.78	0.67	
11	304.4	175.8	136.3	12.67			366				5.12	1.57	3.55	0.77		0.44	
12	312.4	53.9	37.5	5.09			160	+	12.83	12.01	3.96	1.81	2.15	0.69	0.93	0.84	

puncture. All other animals died within a few hours of bleeding and the values, therefore, represent the terminal stages of the disease. Values for various constituents of the blood showed marked changes in the disorder, and while they varied in different animals, the direction of alteration in all constituents with the exception of sugar was constant.

On a basis of blood sugar determinations, the fatal cases of toxemia were divisible into three groups, one of which showed a marked hypoglycemia, another a marked hyperglycemia, and a third approximately normal values. The non-protein nitrogen, urea nitrogen and creatinine values were markedly elevated in all groups and were greatest in the hyperglycemic group. This increase, however, was not a result of blood concentration, as is shown by the disproportionate rise of these and other blood constituents in relation to sugar. The urea partition of the non-protein nitrogen was greater than normal in all animals and in two instances reached the high figure of 83 per cent. Uric acid was also increased but, unfortunately, its value was determined in only one instance.

The sodium chloride value was elevated in one of the two animals in which it was determined. Calcium was decreased and inorganic phosphate increased to such a degree that in all cases the P/Ca ratio approached or was greater than unity. Fat and cholesterol were not uniformly altered, but in general the values were greater than those obtained in healthy animals and in some instances the increase was pronounced. The qualitative acetone test was positive in all cases in which it was made. Serum proteins were slightly increased in one or two instances but generally were somewhat lowered. The albumin and globulin fractions were markedly altered, and in the majority of cases their ratio was reversed.

Preclinical Values.—The course of toxemia in fatal cases is so rapid that consecutive blood studies during the period of clinical illness have so far not been possible. A number of animals, however, that were bled as part of the normal control group subsequently died of toxemia and their blood values give some indication of the preclinical phase of the disorder. The determinations are listed in Table IV, and it should be emphasized that at the time they were made, the animals showed no signs or symptoms of ill health and to all appearance were in excellent condition.

The animal listed first in this series was examined 119 days before the onset of toxic signs and the only abnormalities encountered were a high sugar and low non-protein nitrogen and urea nitrogen values. Likewise the blood of the second animal obtained 75 days before death showed only a slight deviation from the normal manifest in low serum protein value. However, reexamination of this animal 7 days before death disclosed an entirely different blood picture with an increased non-protein nitrogen, low urea nitrogen, increased serum proteins and inversion of the albumin-globulin ratio.

The third animal in the table was examined 36 days before the occurrence of toxemia, and except for low sugar, sodium chloride and creatinine values, the

TABLE IV

Values for Blood Constituents at Various Times Prior to the Onset of Toxemia of Pregnancy

Rabbit No.	Time before death	Condition at bleeding	Whole blood						Serum					Ratios		
			Sugar	Non-protein nitrogen	Urea nitrogen	Creatinine	Sodium chloride	Cholesterol	Calcium	Inorganic phosphate	Total protein	Albumin	Globulin	UN/NPN	P/Ca	A/G
	days		mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	gm. per cent	gm. per cent	gm. per cent			
1	119	R	192.9	22.95	12.7	1.42	445	120	15.77	4.68	5.51	3.58	1.93	0.55	0.29	0.19
2	75	R	145.7	33.57	15.19	1.10	430		15.13	3.6	4.44	3.42	1.02	0.45	0.23	3.3
	7	P	140.1	40.89	8.04	1.69		291.4			5.52	2.49	3.03	0.19		0.8
3	36	R	75	36.36	15.4	0.87	267		14.93		5.49	3.37	2.12	0.42		1.5
4	6	P	129.3	39.1	26.6	1.64	473		13.34	6.16				0.67	0.46	
5	5	P	205.4	31.47	11.8	1.61		85	11.88	3.34	3.86	1.92	1.94	0.37	0.28	0.9
6	2	P	131.2	32.37	20.45	1.68	460	100.4	12.54	5.26	4.14	2.86	1.28	0.63	0.41	2.2

R = resting. P = pregnant.

blood was entirely within the limits of normal. On the other hand, the blood of the next animal, analyzed 6 days before death, showed definite abnormalities with nitrogen retention and a high urea partition.

The fifth animal was examined 5 days before the occurrence of illness and showed high sugar, low serum protein values, and inversion of the albumin-globulin ratio.

The blood of the final animal of this series was analyzed 2 days before death and the suggestive findings were increased urea nitrogen and creatinine with slightly lowered calcium and increased inorganic phosphate values.

It should be noted that the four series of determinations showing most marked abnormalities were made during the gestation period

that terminated in death, while the other determinations were made at various intervals before the fatal pregnancy. The findings in the pregnant group show clearly that the disease may be well advanced before clinical symptoms become apparent. Evidence of a disordered metabolism may be found in the chemical blood picture for as long as a week before the condition is reflected in the animal's behavior, but the degree of variation from normal gives no positive indication of the severity of the condition or the proximity of death.

Blood Chemistry and Reproductive History.—The blood values found in resting animals at considerable periods of time before the clinical

TABLE V

Irregular Values for Blood Constituents of Apparently Healthy Female Rabbits

Rabbit No.	Condition at bleeding	Whole blood					Serum					Ratios		
		Sugar	Non-protein nitrogen	Urea nitrogen	Creatinine	Sodium chloride	Calcium	Inorganic phosphate	Total protein	Albumin	Globulin	UN NPN	P/Ca	A/G
		mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	gm. per cent	gm. per cent	gm. per cent			
1	P	147.8	28.65	8.8	1.33	460	14.16	3.6	5.29	3.93	1.36	0.30	0.25	2.8
2	P	114.5	35.81	10.52	0.96	460	13.61	5.09	4.79	2.75	2.04	0.29	0.37	1.3
3	R	182.8	29.4	11.02	1.5	440	11.59	4.42	4.94	2.53	2.41	0.38	0.38	1.05
4	R	145.7	40.24	22.05	1.61	410	16.98	3.90	7.25	3.57	3.68	0.54	0.23	0.9
5	R	94.3	28.65	11.02	1.94	460	9.31	1.80	3.09	1.65	1.44	0.38	0.19	1.1

R = resting. P = pregnant.

occurrence of the disorder are not markedly abnormal but show definite alteration in one or more particulars. Moreover, the presence of a functional disturbance is reflected in their immediate breeding history. The first two animals in this series were mated shortly after the listed blood values had been determined, and while no abnormalities were noted during gestation, the resulting litter in one instance was born dead and in the other was deserted shortly after birth. Subsequently both were remated and died of toxemia during the ensuing pregnancy. The determinations on the third animal were made just previous to a fertile mating and the gestation period terminated in fatal toxemia.

There have been other instances, however, in which comparable

abnormal values were obtained, but despite numerous pregnancies the animals have continued in apparent health to the present time. These values are listed in Table V. The blood for analysis in the first two instances was taken during the last week of gestation and the remaining determinations were made on resting animals.

Cases of Toxemia with Comparatively Mild Symptoms.—Table VI contains the results of chemical analysis of the blood of four animals obtained during the course of a disturbance which resembled fatal toxemia, both clinically and pathologically, but terminated in recovery rather than in death. With the exception of the absence of acetone bodies, the blood changes are of the same general order but less marked than those noted in fatal toxemia.

In the first instance the manifestations were mild and would have escaped casual observation. The blood showed nitrogen retention with lowered calcium, inorganic phosphate and total serum proteins. $\frac{UN}{NPN}$ was increased but P/Ca and A/G were within normal limits. The signs in the second case were more severe and were accompanied by a slight hyperglycemia, increased non-protein nitrogen with normal urea and a high inorganic phosphate value. It should be noted in this connection that alteration of P/Ca is more closely related to the severity of clinical signs than are changes in other ratios, and an increase as great as that seen in the present instance is usually noted only in fatal cases.

The first analysis of the blood of the third animal in the table was made approximately 10 months before the occurrence of the disorder and showed no irregularity. The second determinations were made while toxic symptoms were marked, but the values were only slightly different from those obtained during health, the only definite alteration being a decrease in serum proteins. Sugar, non-protein nitrogen, urea nitrogen, creatinine and sodium chloride showed small increases. In the final analysis the values for sugar and sodium chloride showed a decided rise. Serum proteins remained low and inorganic phosphate showed a slight increase. Calcium and creatinine were unchanged and all other values were lowered.

It is of considerable interest that the last blood analysis was made at a time when clinical signs were least pronounced and recovery appeared imminent, but the results obtained indicated a metabolic disorder of increasing severity rather than recovery. This animal was killed for pathological study shortly after the last analysis and while a comparison of the values obtained in this animal with those of fatal cases of toxemia would suggest a metabolic disorder of considerably less severity, the lesions were hardly distinguishable from those of fatal cases.

Analysis of the blood of the last animal of this series was begun a few days after the appearance of toxic signs and the last determinations were made shortly

TABLE VI
Values for Blood Constituents in Non-Fatal Cases of Toxemia

Stabilt No.	Whole blood							Plasma	Serum					Ratios					
	Date	Sugar mg. per cent	Non-protein nitrogen mg. per cent	Urea nitrogen mg. per cent	Creati- nine mg. per cent	Uric acid mg. per cent	Sodi- um chlo- ride mg. per cent		Choles- terol mg. per cent	Fat mg. per cent	Acce- tone	Calcium mg. per cent	Inor- ganic phos- phate mg. per cent	Total protein gm. per cent	Albu- min gm. per cent	Globu- lin gm. per cent	UN NPN	P/Ca	A/G
1	1936	147.5	50.55	30.81	1.71	0.89	449			—	11.34	3.00	3.44	2.21	1.23	0.60	0.26	1.8	
		171.4	46.38	15.4	1.43	0.78	469	176.4		—	12.54	8.83	5.66	3.12	2.54	0.33	0.70	1.2	
3	Jan. 17	153	35.3	13.23	1.26		470				12.99	4.8	5.15	3.14	2.01	0.37	0.36	1.5	
	Nov. 6	156.2	36.9	14.07	1.64	0.79	528	113.2	574	—	12.41	4.78	3.14	1.92	1.22	0.38	0.38	1.5	
	Nov. 9	180.8	30.39	12.36	1.68	0.77	554	90.8	644	—	12.41	5.20	3.38	1.87	1.51	0.40	0.41	1.2	
4	Oct. 26	185.2	48.9	20.82	1.74	0.79	498	208.2		—	13.26	5.75	6.72	3.29	3.43	0.42	0.43	0.96	
	Nov. 9	129.2	72.5	35.73	3.44	0.72	459	217.4	430	—	11.83	4.5	5.82	3.28	2.54	0.49	0.38	1.3	
	Nov. 16	139.6	87.86	41.20	3.20	0.74	485	240		—	12.47	6.54	5.82	2.24	3.58	0.46	0.52	0.6	

before death from pneumonia and pulmonary abscess. The first values show an inversion of the albumin-globulin ratio and a rise of all blood constituents with the exception of calcium. In subsequent analyses, the values for sugar, calcium, sodium chloride and total serum proteins tended toward decrease while other constituents continued to increase, and the ratios $\frac{UN}{NPN}$, P/Ca and A/G became increasingly abnormal.

The blood chemistry of this animal was probably influenced by the coexisting infection, and the values obtained are less indicative of the metabolic changes in toxemia than those of the preceding instances.

Pathology

The fatal and milder clinical types of toxemia are generally not distinguishable pathologically and show no consistent difference either in the nature or extent of lesions. The disease is divisible into different pathological types based on alteration of the liver, but these types are apparently not associated with constant clinical or chemical findings. The most striking lesions are found in the liver and kidney as in human toxemia, but changes of interest and of possible pathogenetic importance occur in other organs, and for this reason the gross and microscopic anatomy will be described in some detail.

Gross Morbid Anatomy.—At autopsy all animals that died of the disorder were well nourished and the majority were overfat. The increase in fat generally occurred in normal depots, being greatest in the retroperitoneal region, but in occasional animals an abnormal distribution of fatty pads in the region of the shoulder girdle was noted. Animals that were killed after a prolonged period of illness during which loss of appetite was a pronounced symptom showed considerable muscular wasting without, however, any appreciable diminution in fat stores.

In the majority of cases, whether in pseudopregnant or pregnant animals, and irrespective of the duration of pregnancy, the mammary glands were engorged and actively secreting. The feti in pregnant does were usually dead and on dissection frequently showed liver changes comparable with those observed in the mother. In the majority of instances the placentae were intact and appeared healthy, but in a few cases degenerating placentae without feti were found free in the uterine cavity. This, however, is not an unusual finding in animals killed during gestation and may be due to accidental dislocation or expression of lethal hereditary factors. The ovaries in all cases, in pregnant, pseudopregnant and parturient animals, contained large corpora lutea.

The stomach usually contained food, and the only lesion of the gastrointestinal tract proper was the occasional occurrence of petechial hemorrhages on the surface of the large intestine. Small areas of fat necroses were frequently found in the

mesenteric fat and in some instances there was extensive necrosis of the pancreas and neighboring adipose tissue.

The liver varied in weight from 55 to 210 gm. in animals of different size but averaged 5.5 per cent of the net body weight. The gross appearance was also variable. Frequently, it was uniformly yellow in color or yellow with irregular pinkish red blotches scattered over its surface. Surface markings were absent and all lobular differentiation lost. In such cases the consistency was that of soap and the cut surface imparted a peculiar greasy sensation to the fingers. Rarely, the organ was of a dull greyish brown color with scattered small pale subcapsular areas and fair preservation of lobular markings. The gall bladder was usually filled with dark green bile and showed no abnormality, but occasionally its walls were thickened and its contents granular.

The kidneys were also large and pale and depressed scarred areas were common. In many instances they showed no marked abnormality in appearance, but in others the alteration was striking. The capsule was stretched and stripped easily, revealing a bulging cortex, yellow in color with irregular pink blotches. Section showed an absence of cortical markings with frequent hemorrhagic areas. The urinary bladder was empty in most instances, and in no case of toxemia was sufficient urine found at autopsy to allow a quantitative analysis.

The spleen was always small, averaging less than a gram in weight, and little blood could be expressed from its cut surface.

The pleural and pericardial cavities usually contained an excess of fluid which in many instances was blood-stained and gelatinous in consistency. The lungs were congested and edematous. The heart muscle was flabby and pale and the ventricles were dilated with large amounts of chicken fat clot. Frequently, the right auricle and the vena cavae were devoid of blood and filled with a white milky fluid which on microscopic examination was found to be made up largely of fat globules.

The adrenals were small and their combined weight averaged 0.468 gm. or 0.022 per cent of the net body weight. They were pale yellow in color and extremely soft in consistency. The cortex was wide and frequently contained small adenomata.

The thyroid and parathyroid glands were extremely small and pale. They could be differentiated from the surrounding tissues only with the aid of a magnifying lens, and as a clean-cut separation was not possible, no weights were made.

The hypophysis was always greatly enlarged and averaged 0.044 gm. or 0.002 per cent of the net body weight. In the majority of cases no gross abnormality was noted, but in three instances the posterior half of the gland was found replaced by a large cyst containing colorless fluid.

The brain was wet and edematous, but no other gross abnormality was observed.

Microscopic Examination.—The liver alteration in the great majority of cases consisted of widespread fatty infiltration and degeneration, but in other instances fatty changes were not marked and focal areas of necrosis were the predominating lesion.

Fatty changes were extreme in many cases and involved the entire lobule so that sections resembled adipose tissue. The fat droplets generally tended to be of large size toward the center of the lobule and frequently occupied the entire cell. Nuclei were pushed to the cell margin and were observed in all stages of degeneration. Occasional cells remained intact in this area, but the protoplasm was granular and the nuclei pyknotic. At the periphery of the lobule, the fat droplets were small and were distributed evenly throughout all the cells with a resulting honeycombed appearance. Nuclei were not displaced and degenerative changes were not marked (Fig. 1). Sinusoids throughout the organ were narrowed or obliterated by the encroachment of swollen cells. Occasionally, all cells contained fat droplets exclusively of the large or small type, but the distribution described above was usual (Fig. 2). Special fixation and staining confirmed the presence of fat and showed the complete absence of glycogen. Focal hemorrhages and small discrete areas of necrosis without hemorrhage were occasionally found, but more frequently large irregular areas and sometimes entire lobes were necrotic with thrombosed vessels (Fig. 3).

In other cases fatty degeneration was limited to the periphery of the lobule while the central portion was necrotic or contained patches of necrosis in its peripheral zone (Fig. 4). It should be pointed out in this connection that following the extensive portal fatty change, the periphery of the central zone functioned as the periphery of the lobule and the necrotic areas were, therefore, similar in distribution to those frequently found in eclampsia in man.

In still other instances fatty changes were not widespread, but were limited to focal areas in which all cells were involved irrespective of lobular relationships. In such cases focal necrotic areas were common, widespread throughout the organ and tended toward a peripheral or mid-zonal distribution (Figs. 5 and 6). These areas were sharply circumscribed and adjacent cells showed no alteration. There was no cellular exudation and hemorrhage was rare. The vessels and sinusoids were dilated with blood cells and occasional hyaline thrombi were found. Fibrinous thrombi, however, as often reported in human eclampsia were not observed.

Frequently, both in fatal cases and in animals killed for pathological examination during protracted post partum illness, fibroblastic proliferation into the necrotic areas was noted, implying an older lesion than the clinical history indicated. Moreover, focal and disseminated areas of mature connective tissue were a common finding and may be interpreted as the terminal stage of such a healing process (Fig. 7). If this interpretation is correct, recurrence of toxemia is not uncommon, and as similar areas of fibrosis are found in multiparous does dying of other causes with no previous history of toxemia, it supplies further evidence of the occurrence of asymptomatic cases. The fibrous lesions referred to are readily distinguished from hepatic coccidiosis which is rarely seen in our colony.

Examination of the livers of feti removed from fatal cases showed degenerative and necrotic lesions comparable with those found in the mother.

There was apparently no correlation between the extent of hepatic and renal damage. Kidney lesions were almost exclusively retrogressive in character and

varied from fatty tubular changes to complete cortical necrosis. Active inflammatory lesions were extremely rare.

In many cases the outstanding picture was that of tubular fatty degeneration (Fig. 8). Glomeruli were also affected but to a much less extent. Small areas of hemorrhage were common and rarely hyalin thrombi in arterioles and thrombosed glomeruli were noted. Infrequent inflammatory changes were of the nature of intercapillary glomerulitis with increase of endothelial cells and narrowing of the capillary lumen.

In other instances, cortical damage was extreme with degeneration and necrosis of all elements and advanced dissolution of the entire zone (Fig. 9). The microscopic picture closely resembled that seen in symmetrical cortical necrosis in man and only hazy outlines of swollen glomeruli and granular vacuolated tubular cells with faint pyknotic nuclei remained discernible. Interlobular arteries were thrombosed and in occasional better preserved areas thrombosed glomeruli were recognizable. Medullary structures were intact, but intertubular hyalin changes were common.

Old lesions, including wedge-shaped fibrotic areas, diffuse interstitial fibrosis, scars from focal areas of tubular degeneration with round cell infiltration, together with degenerated and fibrous glomeruli were frequently encountered. The relationship of lesions of this order to past or repeated attacks of toxemia is difficult to evaluate in the present instances, inasmuch as the possibility of parasitic infestation could not be eliminated. Comparison of the lesions found in animals known to have had repeated attacks of toxemia with those encountered in the general population, however, warrants the conclusion that such lesions may be induced by toxemia.

The heart muscle showed intense and widespread fatty changes (Fig. 10). The Malpighian corpuscles of the spleen were small and germinal centers were absent. The pulp was pale staining, washed out in appearance and contained numerous refractile fat globules. In addition to the necrotic lesions previously mentioned, the pancreas showed occasional areas of fibrosis; islands of Langerhans were unusually large and numerous, but hyalin changes were rarely observed.

The fascicular zone of the adrenals was very wide and frequently occupied the entire width of the cortex (Fig. 11). There was an abundance of lipoid throughout the zone; fatty degeneration with necrosis was marked at the inner boundary of the cortex and occasionally was so marked that a fatty necrotic layer entirely separated the cortex from the medulla (Fig. 12). Small hemorrhages were frequently found and cortical adenomata were common.

The thyroid was inactive, follicles were large, lined by low cuboidal epithelium and filled with pale staining colloid.

The hypophysis was studied by means of serial sections in many instances. A good differentiation between granular cells was obtained with Mann's methylene blue and eosin, and contiguous sections were stained with copper hematoxylin to differentiate the cells of the pars intermedia from the basophils of the anterior lobe.

A variety of changes were found in the anterior lobe. Cell counts were not made, but an increase in the number of acidophils was apparent and there was a marked tendency toward grouping of cells with similar staining qualities in different portions of the gland giving the appearance of multiple adenomata. Degranularization of chromophils was a constant feature and was most marked in the vicinity of blood vessels but varied in extent in different sections and in different glands. There were frequently large colloid accumulations in the portion of the lobe adjacent to the pars intermedia. Cells in the vicinity of such accumulations showed degenerative changes and were sometimes necrotic.

The pars intermedia was considerably enlarged in its central portion and the sheath of intermedia surrounding the pars posterior was increased in width. Colloid cysts were numerous and often contained pale eosinophilic concretions resembling the horny pearls of a squamous cell carcinoma. Invasion of the pars posterior with intermedia cells was common, and frequently strands and columns of these cells were found deep in the anterior lobe (Fig. 13). Acidophils were occasionally observed in the intermediate zone, and in one instance a localized area of acidophilic cells resembling an adenoma was found (Fig. 14).

The posterior lobe contained numerous Herring bodies and occasional small dark staining colloid cysts. In instances in which large cysts were found at autopsy, microscopic examination showed them to be limited to the posterior lobe with walls made up of compressed nervous tissue.

The pars tuberalis was hypertrophied and its blood vessels were packed with colloid material to the exclusion of blood cells.

Bacteriological Examination

Blood cultures were made in the terminal stages of the disease in many instances and were uniformly negative. Blood and organ cultures made directly after death have occasionally shown the presence of a few bacteria which were regarded as contaminants or post mortem invaders.

Toxic Factors

The similarity of certain of the pathological changes to those described in poisoning with toxic weeds such as snakeroot (6) suggested the possibility of a contamination in the hay supply of the colony. The hay was, therefore, thoroughly examined, but no toxic weed could be identified. Moreover, a collection of weeds found in the hay was fed exclusively to a number of animals without ill effect.

The water supply of the colony was derived from deep wells and showed no abnormality other than a high mineral content. However, the possibility of the presence of some poison was investigated

by adding scale from the distilling apparatus to the feed of a group of animals. Large amounts of scale were administered in this manner with no resulting illness.

The work on frenching in tobacco plants in the Laboratory of Plant Pathology suggested the possibility of thallium in the water in amounts too small for chemical detection (7), and as the lesions of thallium poisoning are not unlike some of those found in toxemia, a number of animals of the most susceptible group were given small amounts of thallium acetate in their drinking water. Each animal received 3 mg. of thallium acetate daily and the experiment was continued until each had received 50 mg. No ill effects other than a slight loosening of the hair resulted from this treatment and there were no manifestations in any way suggestive of toxemia of pregnancy. Thallium acetate is a cumulative poison and the lethal dose must, therefore, be more than 50 mg. It may be assumed then that had the disease been due to thallium poisoning, the first animal to die would have ingested at least that amount. As each animal drinks approximately 150 cc. of water a day and the first death occurred about 1 month after removal to Princeton, this would mean a concentration of thallium in the water of 0.01 per cent, an easily detectable amount. Tests of the water, however, have all been negative.

DISCUSSION

An accurate and complete interpretation of the problems arising from a study of this disorder is not possible at this stage of the investigation, but many of the findings are suggestive and are worthy of consideration, not only as they affect a distinct disease entity in the rabbit, but also because the disorder apparently bears a relationship to the toxemias of pregnancy in man. The extent of this relationship and evidence pointing to the endogenous origin of the disease in the rabbit will be discussed in subsequent paragraphs, but consideration of etiological factors proper will be deferred to a later paper.

The toxemias of pregnancy in man have been classified on a symptomatic basis and range from slight disturbances to eclampsia, but clear-cut clinical differentiation is not always possible and no characteristic pathological changes distinguish the various types. The disease in the rabbit also presents similar clinical types. The acute

rapidly fatal disorder is analogous to eclampsia, while the less acute non-fatal disturbance is, in many respects, comparable to pre-eclamptic toxemia, and in both species the absence of acidosis and convulsions characterizes the less severe disease. The asymptomatic disorder associated with abortion or desertion and the milder toxic attacks of short duration may be compared to pernicious vomiting of pregnancy, although the characteristic clinical feature is absent due possibly to the presence of a strong cardiac sphincter in the rabbit. Other asymptomatic cases evidenced only by the finding of healed hepatic (or renal) lesions may correspond to the type designated as presumable toxemia in man.

There is considerable controversy regarding the blood chemical findings in eclampsia; the majority opinion is that the sugar may be normal but is often increased and rarely decreased. The non-protein nitrogen, urea nitrogen and creatinine values are not elevated except in cases associated with kidney damage, acetone bodies are increased, uric acid and inorganic phosphate are elevated, calcium is approximately normal, fats and lipoids are not increased more than in normal pregnancy, total proteins are slightly elevated and the albumin-globulin ratio is decreased. The alteration in pre-eclamptic toxemias is less definite and consists mainly in a slight increase of uric acid and a decrease in the $\frac{UN}{NPN}$ ratio.

These findings are in general agreement with those obtained in the rabbit. The blood sugar values varied between hyperglycemia, hypoglycemia and normal levels in animals of different genetic constitution, but the character of the finding was comparatively constant for animals of a given group. In other respects the alteration, although more marked, was in the same direction as noted in man. In non-fatal cases the changes were less pronounced and evidence of acidosis was not found.

The abnormal values found in a number of animals during apparently normal gestation are of particular interest and may be interpreted as evidence of the occurrence of asymptomatic attacks of the disorder. Similar abnormal values were also obtained in healthy resting animals, and it is not improbable that despite the absence of clinical symptoms, these animals also suffered previous attacks of

FIG. 7. Section of liver showing fatty changes with diffuse cirrhosis. This section was taken from a fatal case of toxemia and the animal was known to have had previous attacks of the disorder. Hematoxylin and eosin. $\times 66$.

FIG. 8. Section of kidney showing fatty degeneration of tubular epithelium. Scharlach red. $\times 235$.

FIG. 9. Section of kidney showing degeneration and necrosis of all cortical elements. Hematoxylin and eosin. $\times 110$.

FIG. 10. Section of myocardium showing marked fatty degeneration of muscle fibers. Scharlach red. $\times 235$.

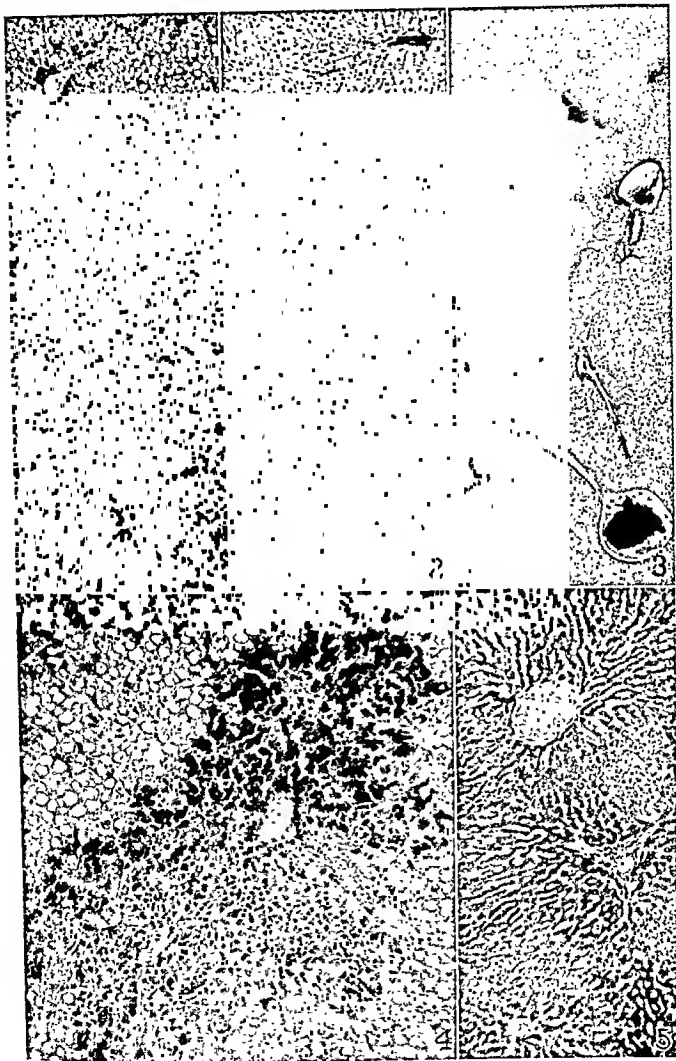
FIG. 11. Section of adrenal showing narrow glomerular zone with an abundance of lipoid in the cells of the zona fasciculata. Hematoxylin and eosin. $\times 110$.

PLATE 26

FIG. 12. Section of adrenal showing a fatty necrotic zone separating the cortex from the medulla. Hematoxylin and eosin. $\times 20$.

FIG. 13. Section of hypophysis showing invasion of the posterior lobe by cells of the intermediate lobe. Mann's methylene blue and eosin. $\times 41$.

FIG. 14. Section of hypophysis showing a localized area of acidophilic cells resembling an adenoma in the pars intermedia. Mann's methylene blue and eosin. $\times 100$.





Photographed by J. A. Carlile

(Greene: Toxemia of pregnancy in rabbit. D)



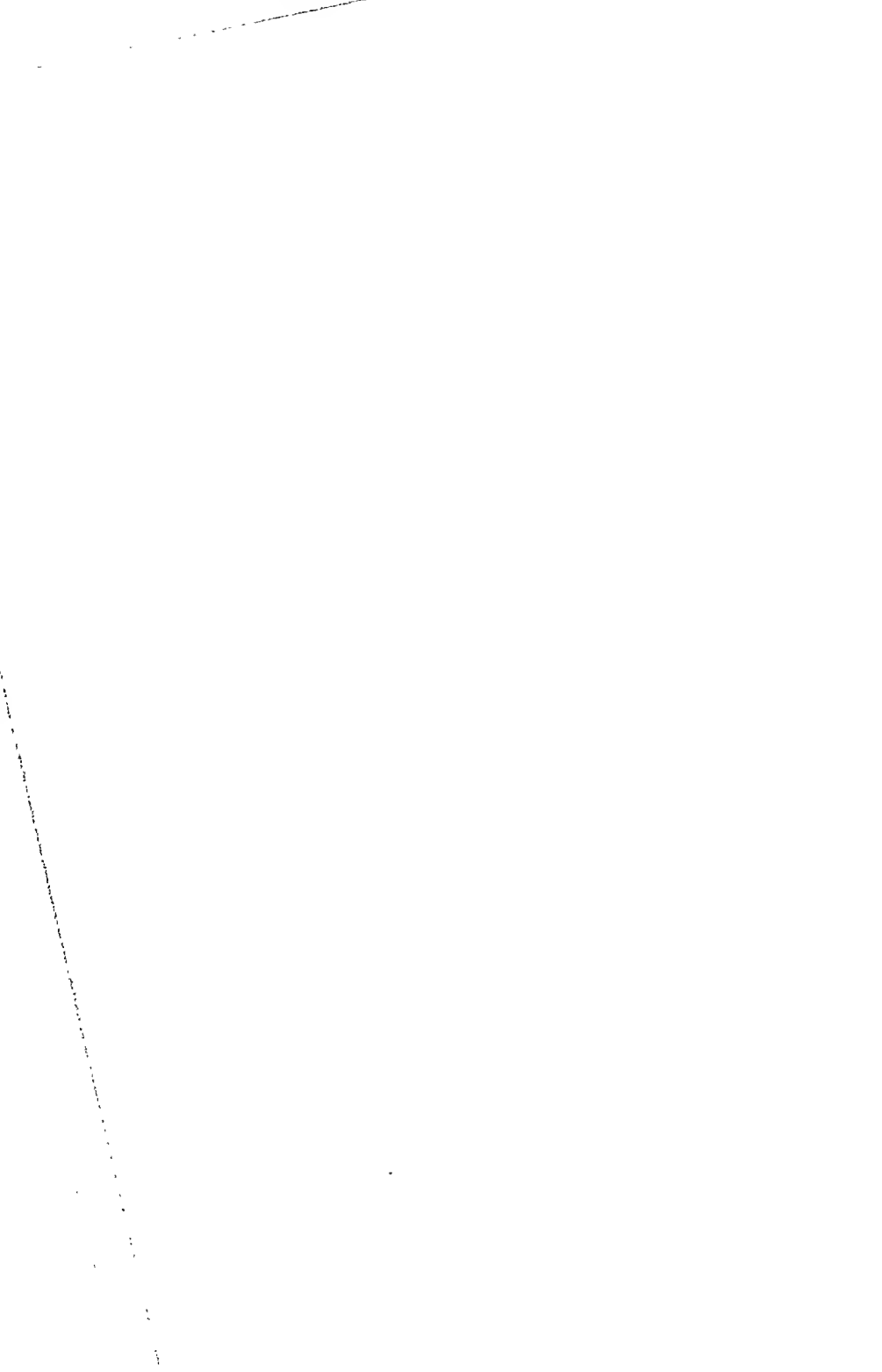
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INFECTIOUS CATARRH OF MICE

I. A NATURAL OUTBREAK OF THE DISEASE

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PLATE 27

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In an earlier report¹ the etiology of a fowl coryza or catarrh of slow onset and long duration was ascribed to certain minute Gram-negative cells descriptively referred to as coccobacilliform bodies. It was of interest to determine whether these bodies, which are unlike any of the known infective agents, were associated with catarrhal states in other hosts. In looking about for suitable material for study an infectious catarrh which met the essential requirement of being readily transmissible was unexpectedly encountered in mice. The nature and etiology of this disease, which it appears has not been previously described or at least not recognized as a distinct entity, are discussed in this and the succeeding papers.

In May, 1935, a group of Swiss mice was brought to The Rockefeller Institute in Princeton, N. J., to replace a breeding colony which had been found² to harbor the virus of acute lymphocytic choriomeningitis. The Swiss breeders together with the accumulated young were held under strict quarantine. Aside from periodic inspections, no one but the attendant came in contact with the colony.

The breeders were divided into groups of 5 females and 1 male. The young were born in the breeding cages and kept there until weaned during the 3rd or 4th week of life. The weaned mice were segregated as to sex in groups of 20-25 individuals. They proved to be particularly poor experimental subjects, being jumpy and irritable. The total population fluctuated somewhat but generally numbered at least 400 breeders and an equal number of young.

In the fall of 1935 we had occasion to examine the nasal passages of several young Swiss mice that had been used experimentally and found a pronounced

¹ Nelson, J. B., *J. Exp. Med.*, 1936, 63, 515.

² Traub, E., *J. Exp. Med.*, 1936, 63, 533.

rhinitis in each animal. Other mice taken directly from the colony likewise showed a nasal inflammation and in addition were observed to make a peculiar chattering noise. An inspection was made of the colony, particular attention being paid to the breeders. At this time there were 75 cages of adults comprising a total of 450 mice. 88 of these mice were chattering when examined. A considerable number of chatterers were also noted among the young mice. During the succeeding month 30 infected mice, 14 of which were breeders, were autopsied. All but one of these mice were chatterers and all but one also showed a rhinitis. A pneumonia was found in 5 animals.

The death rate was low at this time. A considerable number of the older animals, however, had a ragged appearance with ruffed coats. As indicated by an increase in the number of chattering mice the disease continued to spread through the colony. Entering the room where the mice were isolated one was immediately impressed by the simultaneous chattering of many animals. During the latter part of February, 1936, an inspection was made of the young mice. At this time there were 27 cages of mice that had been weaned from 1-7 weeks. In each of the cages, which averaged 20 mice to the cage, there was at least one chatterer, the number varying from 1 to 6. The spread of the disease was so great that it seemed advisable to dispose of the colony and early in March this was done, all of the animals being killed with the exception of a group of 75 that was withheld for observation.

Symptoms of the Disease

Chattering was generally the first apparent symptom of the disease and, while usually coincident with a rhinitis, was sometimes noted in its absence. The nasal inflammation was never accompanied by a discharge either post mortem or during life. Early in the disease, as indicated by observations on recently weaned mice, the chattering was commonly low and intermittent, requiring close inspection for its detection. Snuffling, a distinctly different sound, was also characteristic of the disease. The act of rubbing the front paws over the nose was somewhat more frequent with infected mice than with normal individuals. As the disease progressed the chattering generally became more distinct and regular. The sound of chattering is difficult to describe but somewhat resembles that made by gently clicking the teeth. The sound apparently arises from the lower part of the respiratory tract.

Other symptoms varied considerably with different individuals. A few infected mice showed a loss of weight almost from the onset. Their coats were ruffed, their respiration rapid and shallow, and they

died 3-5 weeks after the appearance of chattering. Many infected mice, however, appeared normal, aside from chattering, for weeks. Their coats were sleek, they gained weight normally, were active, and the females reared their young successfully. Eventually these animals developed the same symptoms and died. Mice in which the disease had progressed slowly over a period of months showed considerable loss of hair, together with a scabby skin and not infrequently marginal necrosis of the ear. Whether these latter symptoms are part of the disease or incidental to it is uncertain.

The Mortality of Naturally Infected Mice

Early in March, 1936, shortly before the colony of Swiss mice was disposed of, a group of 75 animals was removed to a quarantine unit

TABLE I

The Monthly Incidence of Mortality in Naturally Infected Mice

Class	No. of mice	No. of deaths by months										
		Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.
Breeders.....	53	9	8	7	8	4	3	4	4	3	2	0
Young.....	22	0	2	5	3	2	4	1	1	2	0	0
Total.....	75	9	10	12	11	6	7	5	5	5	2	0

and held under observation. This group included 53 infected breeders of both sexes, all of them chatterers, and 22 young mice approximately 6 weeks old. The young mice, which had been kept together since they were weaned, were removed at random from the stock; 3 of them were chatterers, the others symptomless. The breeders were divided into 3 groups which were held in the same cages during the period of observation.

The deaths which occurred among these mice are recorded by months in Table I. The breeders began to die during the 1st month after isolation and by the end of the 4th month 60 per cent had succumbed. How long the breeders had been infected when the period of observation was begun is not known. At the end of the 11th month, when the experiment was discontinued, there was only one survivor in this group.

No deaths were recorded among the young mice until the 2nd month of isolation. The lag here may be accounted for by the fact that only 3 of these animals showed obvious symptoms of the disease when first isolated. At the end of the period 2 of the young mice were still alive.

Because of the condition of the mice when found comparatively few autopsies were made on either the breeders or the young mice. Animals which were examined showed typical manifestations of the disease and invariably a pneumonia. Late symptoms of the disease were obvious in many of the animals prior to death. The mortality incidence which may be regarded as essentially specific was 96 per cent for the entire series of 75 mice during the isolation period of 11 months.

Experimental Transmission of the Disease

Observations on the experimental transmission of the catarrh were delayed by the lack of disease-free animals. Mice of known history were not regularly available until January, 1936. The mice that were finally used came from the same stock as the original colony, in which meningitis had been detected. When this colony was discontinued a small group of pregnant females was withheld. From these³ a nucleus of meningitis-free mice was obtained by selective breeding. Symptoms indicative of the catarrhal disease had never been observed in the parent colony, and young mice from the selected group likewise appeared to be uninfected. Between January, 1936, when transmission experiments were begun and January, 1937, 101 young mice which showed no symptoms of the disease were autopsied. In all animals the middle ears, nasal passages, and lungs were normal. All but 10 of these mice had received a nasal instillation under ether of some material of suspected pathogenicity. The series constitutes an excellent check on the freedom of the mice from the specific disease and on the innocuousness of the instillation procedure when uninfected material was employed.

Mice from the selected colony proved to be highly susceptible to the disease. It was readily and invariably transmitted by the nasal instillation of exudate removed from the upper air passages of naturally infected animals. Maintenance of the disease by the serial passage of exudate from affected to susceptible mice was also accomplished. 6 successive passages were carried out between Oct. 16, 1935, and May 5, 1936, employing a total of 32 mice.⁴ The incidence

³ Under the direction of Dr. Traub.

⁴ A few mice from the selected colony were available in October, 1935.

of disease in this series was 100 per cent. All of the injected mice showed a rhinitis which was accompanied by chattering in 31. 25 of the animals also showed a pneumonia. The middle ears of only 15 of the mice were examined at autopsy; of these, 14 showed an otitis media which was generally bilateral.

In making the nasal instillations pooled exudate, removed at autopsy from the upper air passages of several infected mice, was generally used. The exudate was well mixed with 0.5 cc. of sterile saline. Young mice, 16-20 gm. in weight, were employed. After being deeply etherized, the exudate suspension was administered to them with a capillary pipette, 6-10 drops being placed on the nares. If the animal is well anesthetized the fluid is promptly drawn into the nasal passages without bubbling or sneezing. Usually 4 mice were injected at a time and kept together in the same cage for the duration of the experiment. They were examined regularly after the 1st week, during which time they are commonly symptomless, and were usually brought to autopsy in the 3rd or 4th week after injection.

The symptoms of the disease in experimentally infected mice were identical with those in naturally infected animals. Since most of these mice were killed 3-4 weeks after injection the disease was generally manifested only by chattering. The few mice which did succumb during the period of observation showed the usual loss of weight and ruffling of the hair. Chattering was usually apparent by the 10th to the 14th day after injection. At this time, however, it was commonly light and intermittent, requiring close observation for detection. Later the chattering became louder and more regular.

For some time it was thought that a conjunctivitis was a frequent sign of the disease in injected mice. It was shortly found, however, that uninjected mice removed directly from the stock colony showed symptoms which simulated a conjunctivitis. There was a copious lacrimation with wetting and matting of the hair adjacent to the eye; in some mice there was a distinct ring around the eye. At times the eyelids were swollen and flecked with a white deposit. With a capillary pipette a small amount of chalky fluid could be aspirated. No periorbital swelling was noted. Stained films of the fluid showed numerous epithelial cells but no leucocytes. This condition, the precise nature of which is unknown, may persist for weeks. An occasional injected mouse may, however, show a true conjunctivitis, in

which case the symptoms are identical but films show leucocytes and in addition certain minute Gram-negative coccoid bodies which will be discussed later.

Exudate from the middle ears of mice with an otitis media and suspensions of ground lung from mice with a pneumonia were also infective when administered intranasally. Mice injected in this way showed typical symptoms and postmortem manifestations of the disease.

Postmortem Manifestations of the Disease

The most significant manifestations of the disease were observed only at autopsy. The following summary of the postmortem findings is based on 45 complete autopsies. This group comprises both naturally and experimentally infected animals, the disease being induced in the latter either by the nasal instillation of exudate or by contact.

Forty-three of the infected mice (95 per cent) showed a rhinitis. This condition was never accompanied by a nasal discharge during life. Generally a diagnosis could not be made by macroscopic inspection of the exposed nasal passages but was readily made by aspiration with a capillary pipette. Usually only a small amount of thick mucus which was commonly mixed with blood, as the procedure causes some bleeding, could be removed from the nasal passages of normal mice. From infected animals a copious, semifluid, white exudate of a mucopurulent nature was generally withdrawn. Since only a light suction is necessary for its removal the exudate was rarely mixed with blood. Stained films of the material from normal mice showed scattered epithelial cells and few or no leucocytes, depending on how much blood was present. Films of exudate showed numerous leucocytes together with varying numbers of epithelial cells and strands of mucus. In a few mice the exudate was scanty and contained blood, requiring a microscopic examination for diagnosis. If the material was inflammatory in origin it invariably contained large numbers of leucocytes in nearly every microscopic field. 2 of the mice in this group showed no macroscopic or microscopic indication of a nasal inflammation.

Forty-three of the mice also showed an otitis media, which was bilateral in a but 3 animals. The only outward indication of a middle ear involvement was bulging of the tympanic membrane. This state, however, was rarely observed. Twisting or rotating has never been noted in affected mice from either the select or the original Swiss colony. The presence of exudate in the middle ear cavity which was exposed by cutting through the tympanic bone after removal of the lower jaw, could generally be determined by inspection. The exudate was often copious and typically purulent showing numerous leucocytes microscopically.

A pneumonia was encountered in 35 (77 per cent) of the 45 infected mice. It was usually lobar in distribution; the affected lobe was consolidated, somewhat contracted, and red, gray or mottled in color. Less commonly the pneumonia was patchy, in which case marginal areas of involvement were most often observed. Such areas were sharply demarcated from the normal lung tissue. Abscess formation has never been observed even in advanced cases. Many of the mice in this group were killed 3-4 weeks after injection. In these animals the pneumonia was generally limited to one or two lobes, commonly one of the three right lobes or the small azygous lobe. Adjacent unconsolidated lobes were sometimes definitely emphysematous. From observations on naturally infected mice which were held for a much longer period, it was apparent that the pneumonia was progressive, affecting more and more lung tissue and finally resulting in death of the animal. In mice which had succumbed it was not unusual to find all three right lobes, the median, and part of the large left lobe pneumonic.

Histologically the pneumonia appears to be initially a bronchial involvement with a secondary alveolar extension. Sections show a partial plugging of the bronchi with an exudate composed chiefly of polynuclear leucocytes. There is usually a hyperplasia of the peribronchial lymphoid tissue. The appearance of the parenchyma varies from case to case. The alveoli often contain leucocytes together with red blood cells and large mononuclear cells. In some cases the alveolar walls are distended and there is evidence of fluid in the alveolar spaces.

Communicability of the Disease

The rapid spread of the disease in the original mouse colony indicated that it was readily communicable. It appears probable that direct contact is the significant means of transmission and that indirect contact is relatively unimportant. Cages of normal mice have been kept repeatedly and for considerable periods of time in the same room with many cages of diseased animals without cross infection. No particular precautions were observed in handling the cages. None of the normal mice that were so exposed showed either symptoms or postmortem manifestations of the disease.

That the catarrh was experimentally transmissible by direct contact was shown by 5 experiments in which normal mice were directly exposed to either naturally or artificially infected animals. 27 mice were employed in the 5 experiments. The incidence of infection in these animals was 100 per cent. The onset of the disease was somewhat more gradual than that of mice infected by nasal instillation and its progress was slower. The result, however, was the same; if the experiment was maintained long enough the disease terminated fatally.

In one experiment 6 normal mice were placed in the same cage with 4 naturally infected animals. Chattering was first observed on the 25th day of exposure. 4 of the mice which had shown the usual late symptoms of the disease died during the 18th, 19th, 22nd, and 23rd week, respectively. The only one of these mice which was autopsied showed an extensive pneumonia, a bilateral otitis media, and a rhinitis. The 2 survivors were killed during the 24th week with similar postmortem findings.

In a second experiment 6 normal mice were placed in direct contact with one infected mouse from the preceding group. The exposed mice began to chatter on the 29th day. 5 of these mice which were chattering but otherwise symptomless were killed during the 15th to 19th weeks. All of them showed a rhinitis and an otitis at autopsy. There was no evidence of pneumonia. The 6th mouse was held for further observation. It soon began to lose weight and died during the 26th week. At autopsy there was an advanced pneumonia in addition to a rhinitis and an otitis.

DISCUSSION

The disease of mice described herewith resembles the infectious coryzas of chickens in being essentially an inflammatory response to a specific irritant of surface epithelium. In its slow onset it also resembles the type of fowl coryza which is caused by the minute cells referred to as coccobacilliform bodies. In chickens affected with this type of coryza, however, the inflammatory reaction is generally limited to the nasal passages; the trachea is occasionally involved but the lung is invariably unaffected. In mice the inflammatory reaction is rarely so localized. There is generally an extension of the reaction from its initial locus in the nasal passages to the middle ears and to the lung. In chickens the coryza of slow onset is persistent and a nasal discharge may continue for weeks. It is not fatal, unless complicated by other factors, and if infected birds are held long enough they recover. It appears probable that establishment of the infective agent in mice is always followed by a pneumonia of slow progression which is ultimately fatal.

The mouse disease is attended by relatively few symptoms. Chattering may be the only early means of recognition, but even it is occasionally lacking. Later in the disease constitutional disorder is manifested by loss of weight, inactivity, ruffling of the hair, and altered respiration.

The disease is experimentally transmissible by nasal instillation and

is communicable by direct contact. Under natural conditions it may be epidemic, as was the case in the mouse colony here reported. Experience with another stock of mice, formerly maintained⁵ at The Rockefeller Institute in Princeton, indicates that the disease may also be endemic. Chattering mice were observed from time to time in this colony but were never numerous. At autopsy these mice also showed a pneumonia and an otitis media but their nasal passages were normal. The absence of a rhinitis may have been a factor in limiting the spread of the disease.

Whether this disease is in any way related to the so called mouse influenza recently reported in Germany by Kairies and Schwartzter⁶ cannot be stated at this time. Recognition of these native murine infections is of no little importance in view of the emphasis placed on mice in the study of certain virus diseases of man.

SUMMARY

A natural outbreak of an infectious catarrh in a colony of Swiss mice is reported. The disease was generally characterized by a peculiar chattering sound during life and by a rhinitis, an otitis media, and a pneumonia at autopsy. The pneumonia was slowly progressive and terminated fatally in a high percentage of cases. The mortality in a group of 75 naturally infected mice was 95 per cent over a period of 11 months.

The disease was readily reproducible in susceptible mice by the nasal instillation of exudate from any locus of infection. It was also transmissible by direct contact. In both naturally and experimentally infected animals there was an incubation period of 10 days or more before symptoms were apparent. Recovery from the disease was not observed.

EXPLANATION OF PLATE 27

FIG. 1. A mouse infected with catarrh; showing characteristic posture, ruffling of the hair, and abrasions about the ear.

FIG. 2. A section through the bronchus in a consolidated lobe of the lung of an infected mouse. Stained with phloxin-methylene blue. $\times 105$.

FIG. 3. Distribution of polynuclear leucocytes in exudate from the nasal passages of an infected mouse. Gram stain. $\times 370$.

⁵ By Dr. J. Gowen.

⁶ Kairies, A., and Schwartzter, K., *Centr. Bakt., 1. Abt., Orig.*, 1936, 137, 351.

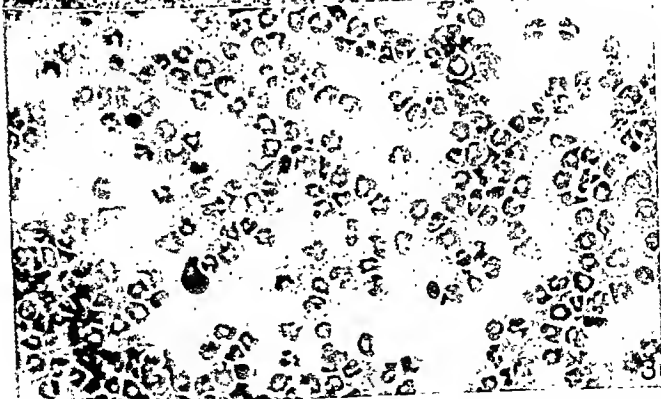




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INFECTIOUS CATARRH OF MICE

II. THE DETECTION AND ISOLATION OF COCCOBACILLIFORM BODIES

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PLATE 28

(Received for publication, March 18, 1937)

The coccobacilliform bodies which are regularly associated with the fowl coryza of slow onset were first observed in films of exudate from the nasal passages of infected birds (1). The isolation of these bodies was hampered by the presence of secondary bacteria in the nasal discharges. It was finally accomplished culturally by the use of exudate from the infraorbital sinuses, where the specific bodies were occasionally found in the absence of free growing bacteria (2). The fowl coryza bodies were found to be cultivable in the fetal membranes of 4 day old fertile eggs and in tissue cultures. They were not demonstrable microscopically in primary blood agar cultures.

The Detection of Coccobacilliform Bodies in Exudate from Infected Mice

Films of nasal exudate from the first observed cases of infectious catarrh in mice showed small Gram-negative cells, in general similar to the coccobacilliform bodies of fowl coryza. These cells were subsequently found in exudate from the nasal passages and the middle ears of practically all diseased mice whether naturally or experimentally infected.

In the preparation of films for examination the exudate was generally diluted with an equal volume of distilled water and thinly spread on a glass slide. The films were Gram-stained, using carbolfuchsin diluted 1:4 with water as the counter-stain. This was applied for only a few seconds.

The cells found in exudate are comparable morphologically with the fowl coryza bodies. They are predominantly spherical but elongated or rod-shaped cells are occasionally noted. Ring forms with a narrow stained rim and an unstained center are also observed. The discrete

cells are often surrounded by a small clear halo. Their size will be dealt with in a later section of this paper. In stained films their outlines are possibly a little less sharply defined than those of the fowl coryza bodies. Like the latter they occur as discrete cells, in pairs, and in small, loosely arranged groupings. Large masses have never been observed. The discrete cell is the characteristic unit of the mouse catarrh bodies. They are predominantly extracellular but may also be found within epithelial cells and polynuclear leucocytes. An intracellular arrangement in leucocytes occurs more often than in fowl coryza. In exudate from the eyes of mice with a conjunctivitis, the coccobacilliform bodies may be found in considerable numbers within very large epithelial cells. They may be so numerous that the cytoplasm of the cell has a granular appearance. Numerically there is a considerable variation from mouse to mouse. In some exudates they are well distributed in nearly every microscopic field. In other specimens their distribution is sparse and many fields may be examined before they are found. In the average exudate the number falls between these extremes but is sufficient to afford a ready diagnosis.

The coccobacilliform bodies were also found in films prepared from the lungs of infected mice. Their certain detection in these films was sometimes made difficult by miscellaneous granules of approximately the same size. Generally there was no possibility of confusion with exudates from the nasal passages and the middle ears. Occasionally the entire film was covered with Gram-negative granules or globules of varying size, some of them being in the size range of the mouse catarrh bodies. In such films absolute identification was impossible. It may be added that these bodies even when present in some numbers can be easily overlooked by an inexperienced observer. Surprisingly few of the many exudates examined showed secondary bacteria, microscopically, in addition to the coccobacilliform bodies. When present they were usually Gram-negative bacilli and Gram-positive cocci. The huge numbers sometimes present in exudate from chickens were never observed in mice, even in cases which had progressed for months.

Isolation of the Coccobacilliform Bodies from the Nasal Passages of Infected Mice

In the first attempts to isolate the coccobacilliform bodies exudate from the nasal passages was employed.

The mice that were used in these and the subsequent isolations were infected by the nasal instillation of exudate. All of them showed typical symptoms and postmortem manifestations of catarrh. The exudates from these mice rarely showed bacteria microscopically but generally contained sufficient organisms, nevertheless, to give a vigorous growth when inoculated into blood agar. Thus, 18 of 26 successive cultures (1.0 cc. of defibrinated horse blood at the base of slanted nutrient agar) of nasal exudate from as many mice showed a macroscopic growth of bacteria. A rather short, non-motile, Gram-negative bacillus was most often encountered. Staphylococci and streptococci were also numerous. Direct isolation of the coccobacilliform bodies from these exudates was impossible. 8 of the blood agar cultures, however, showed no macroscopic or microscopic growth at 24 hours. 5 of the corresponding exudates which had been held meanwhile in the cold room were inoculated into tissue cultures. This medium comprised approximately 75 mg. of finely divided 10 day old chick embryo tissue suspended in 5.0 cc. of Tyrode's solution in 15 mm. test tubes. All of these tissue cultures showed a microscopic growth of minute cells after 24-72 hours' incubation at 37°C. These cells were morphologically similar to those found in exudate.

The Presence of the X Bacillus in Blood Agar and Tissue Cultures of Nasal Exudate

In the early work on the fowl coryza of slow onset there was obtained from a few infected birds a minute Gram-negative organism which formed large compact clumps in the fluid blood at the base of blood agar slants (1). This organism, which was referred to as the X bacillus, is regarded with some experimental justification as an avirulent variant of the coccobacilliform bodies. It may be pointed out that the nomenclature adopted here is one of convenience only and will be corrected at some future time. The names X bacillus and coccobacilliform bodies should at least suffice for purposes of differentiation.

Three of the previously noted blood agar cultures which appeared sterile at 24 hours showed at 72 hours an organism which was morphologically identical with the X bacillus of chickens. 4 of the tissue cultures inoculated with the same exudates later showed large clumps in addition to the discrete coccobacilliform bodies and on transfer to blood agar gave a pure growth of the X bacillus. These 4 cultures were actually a mixture of the two organisms. The 5th tissue culture failed to show large clumps or to grow in blood agar and was obviously pure in respect to the coccobacilliform bodies. It remained so through 7 successive transfers.

The large clumps of the X bacillus are composed of cells which are

equally as small as the coccobacilliform bodies. The individual cells are, however, rarely found outside the clumps. Growth of the organism from mice has been observed only in fluid media. Here it is sparse and films made from a drop of blood may show only 2 or 3 clumps. The clumps vary in size but may attain a diameter of 50μ , being in effect small colonies. A low power objective is useful in finding them. The larger clumps may be so thick and compact that individual cells are apparent only at the periphery. In small groupings the outlines of individual cells may be detectable throughout. The X bacillus is readily maintained by subculturing; one strain has been carried through 60 generations in blood agar at intervals of 3-4 days.

Isolation of the Coccobacilliform Bodies from the Middle Ear Exudate of Infected Mice

The nasal passages having proved to be a poor locus for the isolation of the coccobacilliform bodies, attention was next directed to the middle ears.

The following procedures were adopted in making isolations from the middle ear. The tympanic bone was exposed and seared with a heated spatula. A slit was made on either side of the bone, puncturing the tympanic membrane, with sterile sharp pointed scissors, a separate pair being used for each cut. The resulting V-shaped flap was pushed backwards with sterile forceps, exposing the middle ear cavity. If exudate was present it was removed with a sterile capillary pipette to a tube containing 0.3-0.5 cc. of saline. A drop of exudate was also spread on a slide and the film Gram-stained. If the film showed a fair distribution of coccobacilliform bodies without bacteria, a drop of exudate was transferred to a tissue culture tube and also to blood agar. The inoculated tubes were incubated at 37°C . and a daily microscopic examination made through the 3rd to 5th day.

Twelve tissue culture isolations were attempted from middle ear exudate. The outcome of these isolations is summarized in Table I. Secondary bacteria were not encountered as often as they were in exudate from the nasal passages, only 2 of the specimens showing miscellaneous organisms. In both instances these bacteria were detectable in the tissue cultures but not in the blood agar. Coccobacilliform bodies were present in 10 of the cultures. In 6 of these there was a pure growth which was confirmed by the sterility of the blood agar cultures. 4 of the exudates contained the X bacillus, as indicated by a

typical growth in blood agar, in addition to the coccobacilliform bodies. One exudate showed a growth of miscellaneous bacteria in the tissue culture and a pure growth of the X bacillus in blood agar. With one exudate there was no growth either in the tissue culture or in the blood agar after 7 days' incubation.

It may be added that a single isolation of the coccobacilliform bodies was made from the lung of an infected mouse. This culture also contained the X bacillus.

TABLE I
The Cultural Findings with Middle Ear Exudate

No. of exudate	Growth in tissue culture		Growth in horse blood agar	
	Coccobacilliform bodies	Miscellaneous bacteria*	X bacillus	Miscellaneous bacteria
1	+	+	+	—
2	+	—	+	—
3	+	—	+	—
4	—	—	—	—
5	—	+	+	—
6	+	—	+	—
7	+	—	—	—
8	+	—	—	—
9	+	—	—	—
10	+	—	—	—
11	+	—	—	—
12	+	—	—	—

* Other than the X bacillus.

Some General Properties of the Coccobacilliform Bodies

Cultivation.—Tissue cultures apparently afford optimal growth conditions for active multiplication of the mouse catarrh bodies. In this medium, as indicated by the microscopic examination of stained films, growth of the cells is largely limited to the sedimented tissue. Their morphological appearance here is much the same as in exudate. There appears to be, however, a somewhat greater variation in the size of the cells. Discrete units again predominate. Irregular groupings may also be found, more commonly within masses of disintegrating tissue, but occasionally within intact tissue cells. The mouse catarrh bodies multiply more slowly than those of fowl coryza. Their maximum growth is commonly attained on the 3rd day, although scattered cells may be seen earlier. Like the fowl

coryza bodies, they do not retain their morphological identity for any length of time in tissue cultures.

Growth of the mouse catarrh bodies also occurs in the supernatant of tissue cultures, but it is sparse and cells are found in films only with difficulty. The supernatants of tissue culture media which have been pipetted to other tubes after the tissue has sedimented for 24 hours will support growth in successive transfers with the inoculation of 0.01 cc. of culture. It appears probable that multiplication of the mouse catarrh bodies is not dependent on living cells as such but rather on some particular growth factor which they contain. Recently isolated strains, at least, show neither macroscopic nor microscopic evidence of growth in ordinary nutrient media enriched with blood. The fowl coryza bodies may survive in blood, at first with no microscopic indication of multiplication, and ultimately grow, though sparsely, in it. Whether the mouse catarrh bodies will behave in the same way remains to be determined.

Filterability.—The mouse catarrh bodies will pass through collodion membranes which hold back secondary bacteria present in exudate. In one experiment, employing the Elford ultrafiltration apparatus as designed by Bauer and Hughes (3), pooled exudate from the nasal passages of 3 infected mice was suspended in bouillon and filtered through a collodion membrane with an average pore size of $640\text{ m}\mu$.¹ It had previously been found that this approximate figure represented the limiting pore dimensions for filtration of the fowl coryza bodies. Passage of the mouse catarrh bodies through the membrane was indicated by their growth in tissue cultures inoculated with 0.1 cc. portions of the filtrate. This method should prove useful for their isolation from exudate which contains secondary bacteria. Separation from the X bacillus cannot be made by filtration, however, as this organism is also filterable through the same membranes.

Size.—By the ordinary methods of microscopy it is difficult to determine with precision the size of cells as small as the coccobacilliform bodies. A rough approximation was made with the aid of a micron scale photographed at the same magnification as stained films of the bodies ($\times 920$) and printed on a transparent medium (film). This scale was placed over a photomicrograph and measurements made under a dissecting binocular microscope at a magnification of 10 diameters. The outlines of the cells were somewhat hazy but a reasonable estimation of their dimensions could be made. Measured in this way most of the cells were definitely less than 0.5μ , the average diameter ranging between 0.3 and 0.4μ . There was a scattering of cells a trifle under 0.3 and over 0.5μ . These figures, which are for stained cells (Gram stain), have not been checked by filtration through graded collodion membranes. The demonstrated passage of the bodies through a membrane with an average pore size of $640\text{ m}\mu$, using Elford's (4) factor of 0.75 for the relation of pore size to particle size, is in fair agreement with the measured size of the bodies. It should be emphasized that this filtration was carried out with a

¹ The writer is indebted to Dr. J. H. Bauer of the International Health Division of The Rockefeller Foundation for supplying him with these membranes.

broth suspension containing considerable mucus which tends to clog the filter pores. In size the fowl coryza and mouse catarrh bodies are not far removed from the Paschen bodies of vaccinia; the dimensions of the latter as determined by Elford and Andrewes (4), 0.125μ to 0.175μ , representing the smallest elements of that virus.

SUMMARY

Small Gram-negative cells resembling the so called coccobacilliform bodies of fowl coryza were regularly found in the nasal and middle ear exudate of mice naturally and experimentally infected with catarrh. These bodies were successfully isolated from exudates and cultivated in tissue cultures. There was no microscopic evidence, however, of their multiplication in ordinary nutrient media enriched with blood. They were filterable through collodion membranes with an average pore size of $640\text{ m}\mu$ and, hence, separable from secondary bacteria. The size of the bodies in stained films averaged between 0.3 and 0.4μ .

A second organism cultivable in fluid blood media with the formation of compact clumps and similar to the X bacillus of chickens was also isolated from infected mice.

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EXPLANATION OF PLATE 28

FIG. 1. Scattered extracellular coccobacilliform bodies in nasal exudate.

FIG. 2. An intracellular grouping of the bodies within a leucocyte.

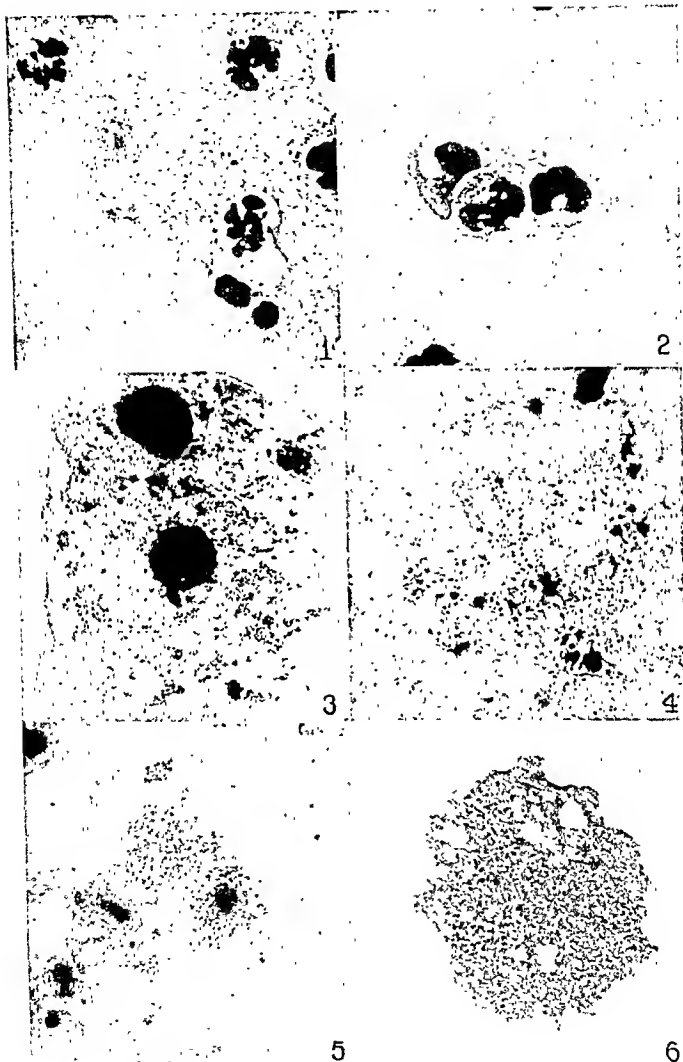
FIG. 3. Intracellular bodies within epithelial cells from the eye of a mouse with a conjunctivitis.

FIG. 4. Extracellular bodies in tissue from a 72 hours old tissue culture of exudate.

FIG. 5. A grouping of coccobacilliform bodies in a mass of disintegrating tissue from a 72 hours old tissue culture.

FIG. 6. A large clump of the X bacillus from a 48 hours old blood agar culture.

The films from which these photomicrographs were made were all Gram-stained and magnified 920 diameters.



Photographed by J. A. Carlile

(Nelson Infectious catarrh of mice, II)

INFECTIOUS CATARRH OF MICE

III. THE ETIOLOGICAL SIGNIFICANCE OF THE COCCOBACILLIFORM BODIES

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The essential features of the slow onset coryza of chickens were reproduced in normal birds by the intranasal injection of tissue culture suspensions of the coccobacilliform bodies (1). The experimentally initiated coryza was serially transmissible by injection and communicable by direct contact. One discrepancy was noted, however, in comparison with the natural disease. Birds injected with the coccobacilliform bodies in pure culture often failed to show a nasal discharge during life although an inflammation of the nasal passages was present at autopsy.

In this paper similar experiments are reported on the infectivity of the minute bodies which were regularly associated with murine infectious catarrh and were successfully isolated and grown in tissue culture (2, 3).

General Experimental Procedures

Suspensions of primary tissue cultures and subcultures which showed coccobacilliform bodies microscopically were administered by nasal instillation to normal etherized mice.

The supernatant was withdrawn from 48-72 hour old tissue cultures; the sedimented tissue removed to a glass tissue grinder, well triturated, and the residue added to approximately 0.5 cc. of the supernatant. Mice were deeply etherized and a few drops of the turbid tissue suspension placed on the nares. 3 to 5 mice, 15-20 gm. in weight, were injected with each culture. The mice were kept together in the same cage for the duration of the experiment and examined frequently. They were killed after 3-6 weeks and autopsied, particular attention being paid to the nasal passages, the middle ears, and the lungs. If exudate was present, films were made and Gram-stained.

The Infectivity of Tissue Culture Transfers in the Presence of the X Bacillus

In the first 2 experiments, 2 lots of 5 mice each were injected intranasally with suspensions of 5th tissue culture transfers. They were killed at intervals between the 3rd and 5th weeks thereafter and examined. None of the mice had chattered or shown any suggestive symptoms during the period of observation. At autopsy they were all normal, there being no indication of inflammation in any of the usual loci.

The 2 tissue cultures used in these experiments contained the X bacillus, presumably in addition to the coccobacilliform bodies. The presence of the former organism was indicated by compact clumps in tissue cultures and in horse blood agar transfers. That coccobacilliform bodies were also present was inferred by the detection of discrete cells. The presence of discrete cells is not, however, strictly diagnostic, differentially, as is the compact grouping.

The Infectivity of Primary Tissue Cultures

The injection of primary tissue cultures was then tried, with quite different results. 11 primary cultures isolated from as many mice were used in these tests. 2 of the cultures were inoculated with suspensions of lung tissue, the others with suspensions of exudate from the middle ear. 6 of the cultures contained the X bacillus in addition to the coccobacilliform bodies; 5 showed a pure growth of the latter. The incidence of catarrh in the 40 mice that were injected with these cultures was 100 per cent. Although the symptoms and postmortem indications of the disease fluctuated considerably from mouse to mouse, all of the injected animals displayed inflammatory manifestations in at least one of the usual loci.

The results of these 2 groups of experiments were open to a number of interpretations. The coccobacilliform bodies might be etiologically significant but subject to a rapid loss of virulence with cultivation. The presence of the X bacillus in the same culture with the coccobacilliform bodies might ultimately result in a depression of their virulence or growth. The coccobacilliform bodies might be of no etiological significance, the infectivity of initial cultures being referable to some unknown agent present in the inoculated exudate.

Since the infective primary cultures contained no cultivable organisms aside from the coccobacilliform bodies and the X bacillus, ordinary bacteria could be eliminated with reasonable certainty. That the infectivity of these cultures was referable to a filterable virus was possible. The tissue culture medium used for cultivation of the coccobacilliform bodies would probably be an unfavorable environment for the increase of a virus. 2 filtration experiments were carried out to test this possibility. Broth suspensions of 2 exudate samples were rapidly filtered through short Berkefeld V candles and the filtrates injected as usual in 2 groups of 5 mice each. Both filtrates were innocuous, none of the injected mice showing either symptoms or post-mortem signs of the disease.

The Comparative Infectivity of Primary Tissue Cultures and of Exudate Suspended in Tyrode's Solution

The improbability that a second infective agent was present in exudate was borne out by another experiment in which mice were injected with suspensions of initial tissue cultures and with exudate subjected to the same dilution and incubation in Tyrode's solution.

Approximately 0.05 cc. of exudate from the middle ears of 2 infected mice was suspended in 0.5 cc. portions of saline and 0.1 cc. of the mixtures added to a tissue culture and to a tube containing 5.0 cc. of Tyrode's solution. A horse blood agar slant was also inoculated. The 2 series of 3 tubes were incubated at 37°C. for 3 days. The Tyrode controls remained clear. The 2 tissue cultures showed coccobacilliform bodies without compact clumps. The horse blood agar cultures were sterile, with no microscopic evidence of growth. On the 3rd day 2 lots of 3 mice were injected with suspensions of the tissue cultures and the same number with the exudate suspensions in Tyrode's solution.

The results of this experiment are presented in Table I. All of the mice in the 2 series of 3 which received a nasal instillation of 72 hour old tissue culture suspensions showed manifestations of catarrh at autopsy. The 6 mice which were injected with the same exudate subjected to the same dilution and incubation in Tyrode's solution were normal at autopsy. The amount of exudate which the mice received was roughly 0.001 cc.

The Infectivity of Primary Tissue Cultures and Subcultures Compared with Primary Blood Agar Cultures and Subcultures

A variation of the preceding experiment using horse blood at the base of slanted agar in place of Tyrode's solution was also carried out. Third subcultures were tested in addition to the primary ones.

Tissue cultures containing 5.0 cc. of Tyrode's solution and nutrient agar with 1.0 cc. of defibrinated horse blood at the base were inoculated with 0.01 cc. of

TABLE I

The Reaction in Mice Injected with Primary Tissue Cultures and with Exudate Suspended in Tyrode's Solution

Material injected	Mouse No.	Reaction in injected mice			
		Chattering	Rhinitis	Otitis	Pneumonia
Exudate 193 in tissue culture	1	+	+	+ U*	+
	2	+	+	+ U	+
	3	—	—	+ U	+
Exudate 194 in tissue culture	4	—	—	+ B	+
	5	+	+	+ B	+
	6	+	—	+ U	+
Exudate 193 in Tyrode's solution	7	Normal throughout			
	8	" "			
	9	" "			
Exudate 194 in Tyrode's solution	10	" "			
	11	" "			
	12	" "			

* In this and the following tables U signifies a unilateral, B a bilateral otitis.

saline suspensions of middle ear exudate (approximately 0.05 cc. of exudate in 0.3 cc. of diluent) from each of 2 infected mice. Both series of cultures were incubated at 37°C. for 72 hours. The tissue cultures showed typical coccobacilliform bodies without compact clumps. The blood agar cultures were sterile, there being neither microscopic nor macroscopic indication of growth. Suspensions of the 72 hour old tissue cultures and undiluted blood from the blood agar cultures were injected as before. Third subcultures in both media were similarly tested for infectivity. The microscopic findings with these cultures were identical with the primary ones.

The results of this experiment are summarized in Table II. All of the 12 mice which were injected with primary and tertiary sub-

TABLE II

The Reaction in Mice Injected with Tissue and Blood Agar Cultures of Exudate

Material injected	Mouse No.	Reaction in injected mice			
		Chattering	Rhinitis	Otitis	Pneumonia
Exudate 204 in primary tissue culture	1	+	+	+ B	+
	2	+	+	+ U	+
	3	-	+	+ U	+
Exudate 204 in 3rd tissue subculture	4	+	-	+ U	+
	5	+	+	+ U	-
	6	+	+	+ B	+
Exudate 205 in primary tissue culture	7	+	-	+ B	+
	8	+	+	+ B	-
	9	-	-	+ U	+
Exudate 205 in 3rd tissue subculture	10	+	-	+ B	-
	11	-	+	+ B	+
	12	-	+	+ B	-
Exudate 204 in primary blood agar culture	13	-	-	-	-
	14	-	-	-	-
	15	-	-	-	-
Exudate 204 in 3rd blood agar subculture	16	-	-	-	-
	17	-	-	-	-
	18	-	-	-	-
Exudate 205 in primary blood agar culture	19	-	-	-	-
	20	-	-	-	-
	21	+	-	+ U	+
Exudate 205 in 3rd blood agar subculture	22	-	-	-	-
	23	-	-	-	-
	24	-	-	-	-

cultures of the coccobacilliform bodies showed postmortem manifestations of catarrh. One mouse only of the 6 which received primary horse blood agar cultures also displayed unmistakable signs of the dis-

case at autopsy. The remaining 5 mice in this series and the 6 which were injected with tertiary blood agar cultures were all normal throughout.

Of the many different groups of 3 to 5 mice which had been injected this was the only instance in which all the individuals of a group failed to react in the same way. It seems reasonably certain that the one positive mouse in the blood agar culture series did actually acquire the disease as a result of the injection. The inoculum which this animal received may have contained a small bit of unsuspended exudate in which the infective agent survived despite the unfavorable environment.

The results of this and the immediately preceding experiment were essentially the same and indicate that the infectivity of exudate is retained only in a medium which affords suitable conditions for multiplication of the coccobacilliform bodies. In neither experiment was there a suggestion that any agent other than the coccobacilliform bodies was present in the exudate.

The Infectivity of Tissue Culture Transfers of the Coccobacilliform Bodies in the Absence of the X Bacillus

Three of the tissue culture strains of the coccobacilliform bodies employed in the 2 preceding experiments were carried through additional transfers and their infectivity tested by nasal instillation in mice. These subcultures, unlike the earlier ones which proved to be uninfected, were pure in respect to the coccobacilliform bodies. Compact clumps indicative of the X bacillus were not present in tissue cultures and blood agar transfers were sterile. The results of these tests are brought together in Table III. Strain 193 was carried through 12 subcultures, the 2nd, 5th, and 12th being tested. Strains 204 and 205 were each carried through 7 subcultures, the 3rd and 7th being tested. The transfers were made at intervals of 2-3 days. All of the mice injected with these several subcultures showed typical manifestations. These tests show that tissue cultures inoculated with exudate and showing no demonstrable agent other than the coccobacilliform bodies may be infective after 12 successive transfers.

Manifestations of the Catarrh Produced by Tissue Culture Injection

The ante- and postmortem signs of the disease in mice infected by the nasal instillation of tissue culture suspensions were much the same as those in mice infected by the injection of exudate. Chattering was

TABLE III

The Reaction in Mice Injected with Pure Tissue Culture Transfers of the Coccobacilliform Bodies

Designation of culture	Mouse No.	Reaction in injected mice			
		Chattering	Rhinitis	Otitis	Pneumonia
2nd subculture No. 193*	1	+	+	+ B	+
	2	+	+	+ U	+
	3	+	+	+ B	-
	4	+	+	+ B	+
5th subculture No. 193	5	+	-	-	+
	6	-	+	+ B	+
	7	-	-	+ B	-
	8†	+			
12th subculture No. 193	9	+	-	+ B	+
	10	+	-	-	+
	11	-	-	+ U	-
	12	-	-	+ B	-
7th subculture No. 204	13	+	+	+ B	+
	14	-	+	+ B	-
	15	-	+	+ B	-
7th subculture No. 205	16	+	+	-	+
	17	+	+	+ U	-
	18	-	+	+ B	-

* For the other cultures in this series see Tables I and II.

† This mouse died and could not be examined.

apparent during life, and at autopsy a rhinitis, an otitis media, and a pneumonia were encountered. A conjunctivitis was never observed. Coccobacilliform bodies were invariably demonstrated in films from one or another locus of inflammation. All of the characteristic signs of the disease occurred, however, somewhat less frequently in these

mice. This difference is indicated in Table IV. The number of mice which failed to develop a rhinitis (approximately a third) was particularly striking. The disease obviously progresses more slowly in mice infected with cultures and the observed differences may be referable in part to an insufficiently protracted period of observation.

Communicability of the Catarrh Produced by Tissue Culture Injection

The communicability of the experimentally produced catarrh was determined by 2 contact experiments. In both tests a mouse infected by the nasal instillation of a tissue culture suspension, indicated by chattering, was placed in the same cage with 4 normal mice. The infected mouse in the first group died on the 22nd day of observation

TABLE IV

The Incidence of Catarrhal Manifestations in Mice Injected with Exudate and with Tissue Cultures

Material injected	No. of mice	Percentage showing			
		Chattering	Rhinitis	Otitis	Pneumonia
Exudate.....	45	95	95	95	77
Tissue culture.....	64	68	59	89	67

and at autopsy showed all of the usual manifestations of the disease including rhinitis. The exposed mice began to chatter during the 5th week and were killed at the end of the 15th week. At this time they all showed obvious symptoms in addition to chattering. The usual inflammatory reactions were encountered at autopsy.

The results of the second experiment were strikingly different. By chance, an infected mouse with a normal upper respiratory tract was selected as the contact. The 5 mice were kept together in the same cage for 10 weeks. Throughout this period the infected mouse chattered but the exposed animals remained normal. At autopsy the contact mouse showed only a pneumonia. The 4 exposed mice, which had gained weight continuously, were normal with no detectable manifestations of catarrh in any of the usual loci.

DISCUSSION

The results of the preceding experiments afford reasonably conclusive evidence that infectious catarrh of mice is caused by the minute elements, termed coccobacilliform bodies, which are regularly present in exudate. Primary tissue cultures of exudate were consistently infective. Excluding tissue components the only formed elements which were microscopically demonstrable in all these cultures were the coccobacilliform bodies. In view of the innocuousness of exudate passed through coarse Berkefeld filters it was unlikely that an agent below the limits of microscopic vision was present in the inoculum. Some of the primary cultures contained the so called X bacillus in addition to the coccobacilliform bodies. Transfers made from such cultures were usually uninfected, whereas transfers of cultures which contained only the coccobacilliform bodies were pathogenic. Not only was the X bacillus uninfected itself, but by its continued growth in transfers it appeared to bring about a complete loss of activity in cultures which were initially infective. Tissue cultures which contained only the coccobacilliform bodies remained infective through as many as 12 transfers. By the practical elimination of any additional agent in these cultures, it appears justifiable to conclude that their infectivity was referable to the coccobacilliform bodies.

The disease produced by the coccobacilliform bodies, in tissue culture suspensions, approached the natural disease but showed certain quantitative differences. Its onset and progress were often retarded. The same manifestations were observed at autopsy but were somewhat less frequent than in naturally infected animals. The incidence of rhinitis showed a decrease of approximately one-third. This observation was of interest in connection with the naturally encountered endemic type of the disease. Infected mice from a colony in which only sporadic cases were observed also failed to show rhinitis. It was suggested that this circumstance might have a bearing on the communicability of the disease (2). The contact tests reported in this paper give some support for this suggestion. The disease was transmitted to normal mice by contact with an infected animal with rhinitis. It was not transmitted, however, by a diseased mouse with a normal upper respiratory tract.

The observations reported in these papers indicate that the minute entities referred to as coccobacilliform bodies do occur in association with a catarrhal state in hosts other than fowl. The coccobacilliform bodies found in chickens and mice, respectively, are far from being identical but are sufficiently similar to suggest a close relationship.

The disease termed mouse influenza by Kairies and Schwartz (4) was believed to be caused by an influenza-like bacillus which was readily cultivable. The writers note, however, that in rapidly growing fluid cultures small forms were present which were filterable through membranes with a pore size of 0.4–0.6 μ . They regarded these small bodies as a filterable form of the bacillus. These small forms may or may not bear a relation to the coccobacilliform bodies. In the case of the latter it can be said with some assurance that they are not akin to larger readily cultivable bacteria.

SUMMARY

Evidence is presented that the etiology of infectious murine catarrh is specifically referable to the coccobacilliform bodies. The disease was regularly produced in normal mice by the nasal instillation of primary tissue cultures. In the presence of the X bacillus, transfers of primary cultures were usually uninfected. Pure cultures, however, retained their pathogenicity through as many as 12 transfers.

The onset and progress of the experimental disease were somewhat retarded in comparison with the natural disease, but in general there was a close parallel. Mice injected with cultures did, however, show a significant decrease in the incidence of rhinitis. Transmission by direct contact was demonstrated in the presence of a rhinitis but not in its absence.

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THE ACTION OF IMMUNE SERUM ON HUMAN INFLUENZA VIRUS IN VITRO

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The mechanism by which immune serum inhibits the disease-producing function of filterable viruses has been subjected to repeated study by different workers and with different viruses. In most instances amounts of serum just, or not quite sufficient to "neutralize" the selected dose of virus have been employed. Bedson (1), Todd (2), Andrews (3), and Craigie and Tulloch (4) have shown that by dilution of such a mixture it is possible to recover active virus. It has also been shown that a serum-virus mixture may produce no evidence of infection when administered to susceptible animals by one route but may produce typical infection if administered by another route (2, 3, 5-7). On the basis of these results the conclusion has been generally drawn that no union between immune substance and virus occurs *in vitro*. Sabin (8) reached similar conclusions by demonstrating that virus could be recovered from a neutral serum-virus mixture by high speed centrifugation, and Long and Olitsky (9) have recovered vaccine virus from immune animals by cataphoresis.

In most of these instances, however, the treatment of virus with immune serum has resulted in a reduction of the infectious titer of the virus suspension when compared with the effect of normal serum. The reduction of virus titer has been most pronounced in the presence of an excess of immune serum or when a period of several hours' incubation has preceded dilution of a mixture. Certain workers have, therefore, postulated that some type of reaction, agglutination or inactivation, between the immune substances and the virus occurs *in vitro* (1, 10-13).

In studies with human influenza virus cultivated in chick embryo-Tyrodé culture medium, it was noted, in agreement with observations

of others, that the addition of immune serum to the medium before the introduction of virus resulted in a failure of the virus to survive or multiply. The fact that the virus was well adapted to growth under artificial conditions which readily lent themselves to a study of the influence of immune serum upon virus in the presence of cells suggested further investigation of the problem. The present report deals, therefore, with certain experiments which have a distinct bearing upon the problem of the reactions between immune serum and virus *in vitro*.

Materials and Methods

The strain of human influenza virus employed was the PR8 strain which has been maintained in chick embryo tissue culture medium through 200 consecutive transfers during a period of 16 to 18 months (14). The strain of swine influenza virus was the S15 strain,¹ transferred to tissue culture medium in which it has been maintained through 150 transfers. The tissue cells consisted of minced 12 day chick embryos in a concentration such that 5 cc. of culture medium contained approximately 200 mg. of minced embryo tissue. The immune sera were obtained from ferrets convalescent from infection with PR8 human influenza virus or from rabbits immunized by means of intraperitoneal inoculation with the virus. Tests for the presence or absence of active virus in a given mixture were made by inoculation of the material to be tested into the nasal passages of anesthetized susceptible mice. The lungs of all the mice, either at the time of death or at the end of the observation period of 9 to 10 days, were examined for gross pathological lesions characteristic of infection by influenza virus.

Centrifugation of cells was performed in the ordinary centrifuge at 2000 to 3000 revolutions per minute. The high speed centrifuge used for sedimentation of serum-virus mixtures was the air-driven one described by Bauer and Pickels (15). In each instance the use of the term "washing" indicates that the sedimented material was resuspended in the wash fluid and this suspension again subjected to centrifugation.

The Effect of Immune Serum upon Tissue Cells

Since only three immunologically active components—virus, cells, and serum—are present under the conditions of study, the effect of immune serum in preventing virus infection must be exerted in one of three ways: the serum must act on the tissue cells, on the virus, or only in the presence of both virus and cells. Sabin (16) has interpreted his results to indicate that the immune substances in serum act primarily upon the tissue cell rather than upon the virus. An experi-

¹ Obtained through the kindness of Dr. R. E. Shope.

ment was conducted, therefore, to determine whether the exposure of the tissue cells to immune serum had any effect, persisting after washing, upon the growth of influenza virus subsequently added.

Two flasks containing 4.0 cc. of Tyrode solution and 0.2 cc. of minced 12 day chick embryo were prepared. To one was added 0.5 cc. of immune ferret serum; to the other, 0.5 cc. of normal ferret serum. After incubation at 37°C. for 24 hours, the cells from each flask were removed by centrifugation at 3000 R.P.M., washed twice with about 8 cc. of Locke's solution, and then reintroduced into flasks containing 4.5 cc. of Tyrode solution. To each flask was added 0.5 cc. of influenza culture virus and the cell-virus suspensions were incubated at 37°C. for 48 hours. The cells were again removed by centrifugation and disrupted by grinding in Locke's solution. Both the suspensions of ground cells and the supernatant fluids of the cultures were tested for their virus content by means of mouse tests.

TABLE I

Growth of Virus in Cultures Containing Cells Treated with Normal or Immune Serum

Cells treated with serum; incubated 24 hours; washed twice; returned to culture medium; and virus then added.

A. Cells treated with immune serum	Cells Supernatant	d++++	d++++	d++++	d++++
		d++++	d++++	d++++	d++++
B. Cells treated with normal serum	Cells Supernatant	d++++	d++++	d++++	d++++
		d++++	d++++	d++++	d++++

d++++ = mouse died before 10th day with complete pulmonary consolidation.

It is seen from Table I that multiplication of the virus was supported as well by the cells previously exposed to immune serum as by those treated with normal serum. No evidence was obtained to indicate that immune substances were retained by the cells in sufficient concentration to interfere with the survival or multiplication of the virus.

The Effects of the Order of Addition of Serum and Virus to Cells upon the Subsequent Survival and Multiplication of the Virus

Rivers, Haagen, and Muckenfuss (17), Andrewes (18), and Sabin (16) found that vaccine virus added to a suspension of minced rabbit testis before the addition of immune serum, not only survives but multiplies and produces inclusion bodies in the cells, whereas

when the immune serum is added before the virus the latter neither multiplies nor produces inclusion bodies. In the present study, while using immune serum of low titer, it was also observed that the order in which serum and virus are added to cells distinctly affects the survival of virus in the cells. When virus was added to the tissue culture medium before immune serum, virus was found to be present within the cells after a period of incubation of 24 hours at 37°C. The following protocol illustrates an experiment of this type.

Two flasks (A and B) containing 3.5 cc. of human influenza virus and 0.2 cc. of minced 12 day chick embryo were prepared. They were allowed to stand at room temperature for 1 hour. To flask A was added 0.5 cc. of PR8 immune rabbit serum and to flask B, 0.5 cc. of normal rabbit serum. The cultures were then incubated at 37°C. for 24 hours.

Two additional flasks (C and D) were prepared as follows:

- Flask C. 3.0 cc. human influenza virus
0.2 cc. minced embryo
2.0 cc. PR8 immune rabbit serum
- Flask D. 3.0 cc. human influenza virus
0.2 cc. minced embryo
2.0 cc. normal rabbit serum

The constituents of flasks C and D were added in the order given. The virus and cells were allowed to stand together at room temperature for several minutes before serum was introduced. After the addition of serum, the two flasks were incubated at 37°C. for 1½ hours.

At the end of the incubation period the contents of each flask were separately centrifuged at 2000 R.P.M. for 30 minutes, the supernatant fluid decanted, and the cells washed twice in physiological salt solution. The washed cells from each flask were ground in 2.5 cc. physiological salt solution and the virus content determined by mouse tests. The supernatant fluids were centrifuged in the air-driven centrifuge at 14,000 R.P.M. for 3 hours. Each sediment, drained free of supernatant fluid, was resuspended in 5.0 cc. of physiological salt solution and the virus content of the suspension was titrated in mice.

The results shown in Table II demonstrate that after 24 hours' incubation at 37°C. in the presence of specific immune serum, no active virus was present in the supernatant fluid although still present in considerable amounts in the cells. Nevertheless, these cells contained less virus than those incubated in the presence of normal serum. That the absence of virus in sediments obtained by centrifugation of the supernatant fluid was not due to a residuum of free immune serum in

the fluid is evident, since the serum diluted 1 in 100 failed to neutralize human influenza virus and under the conditions of the experiment a dilution of serum would be reached outside the effective range. It is interesting to note as well that swine influenza virus used as an additional control was apparently unaffected by the PR8 immune serum.

In a second type of experiment the same immune rabbit serum as used in the previous experiment was incubated with human influenza virus for 30 minutes at 37°C. prior to the addition of embryonic cells. Under these conditions there is equal opportunity for free antibody and free virus to act upon the cell.

To a flask containing 4.0 cc. of Tyrode solution was added 0.5 cc. of culture virus and 0.5 cc. of immune rabbit serum; to another, 0.5 cc. of virus and 0.5 cc.

TABLE III

Effect of Weak Serum When Incubated with the Virus for 30 Minutes before Addition of Cells

Serum and virus incubated 30 minutes. Cells added and incubated 2 hours. Cells removed, washed twice, and added to medium containing fresh cells. Incubated 24 hours. Cells ground in supernatant fluid and tested in mice for virus content.

	Degree of pulmonary consolidation			
	.0	0	0	0
PR 8 immune rabbit serum.....	d++++	d++++	d++++	d++++
Normal rabbit serum.....	d++++	d++++	d++++	d++++

of normal rabbit serum. Both were incubated at 37°C. for 30 minutes, and then to each was added 0.2 cc. of minced 12 day chick embryo. After 2 hours' incubation at 37°C., the cells from each were removed by centrifugation at 2000 R.P.M., washed twice in Locke's solution to remove excess serum, and then added to flasks containing 4.5 cc. of Tyrode solution and 0.2 cc. of fresh embryonic cells but no serum. The secondary cultures were incubated at 37°C. for 48 hours, the cells removed, ground with sand, and resuspended in the supernatant fluid. The resultant suspensions were tested for virus by inoculating them into mice.

The results given in Table III show that under these conditions virus exposed to the action of immune serum failed to multiply or survive in the cells.

The results of the foregoing experiments, in which serum of low

neutralizing titer was used, agree with the conclusions of other investigators, that when virus is added to cells before immune serum the virus survives in the cells although in a lesser concentration than in the presence of normal serum. On the other hand, when virus was first mixed with immune serum and added to the cells after incubation, no evidence of survival or multiplication of the virus was obtained. In fact, the virus appeared to be completely inactivated.

The Effect of Serum of High Neutralizing Capacity

The preceding observations suggested that even with a serum of comparatively low antiviral titer, a detrimental effect upon multiplication of virus was exerted. In the following experiments convalescent ferret serum was used. The titer of this serum was such that a dilution of 1 in 200 mixed with undiluted tissue culture virus prevented the development of pulmonary lesions in mice inoculated with the mixture intranasally.

Using this serum, the experiments concerned with the effect of immune substances added to the cells before or after the introduction of virus were repeated.

To one of four flasks, each containing 0.2 cc. of minced 12 day chick embryo in 4.0 cc. of Tyrode solution, was added 0.5 cc. of PR8 immune ferret serum, and to another 0.5 cc. of normal ferret serum. The other two flasks each received 0.5 cc. of PR8 strain of culture virus. All four flasks were incubated at 37°C. for 1 hour. Then to each of the first two was added 0.5 cc. of virus; to the third, 0.5 cc. PR8 immune ferret serum; and to the fourth 0.5 cc. of normal ferret serum. All four were again incubated at 37°C. After 24 hours the cells were separated by centrifugation, washed twice in Locke's solution, and ground in 5.0 cc. of Locke's solution. Each suspension was tested for virus by the intranasal inoculation of mice.

The results are given in Table IV. Under these conditions no virus was demonstrable in the washed cells after 24 hours' incubation in the presence of immune serum, regardless of whether the serum was added 1 hour before or 1 hour after the cells were exposed to the virus.

That the action of the immune serum is not only on the virus in or adherent to the cells, but is also exerted on that contained in the fluid portion of such "tissue cultures" is evident from the results of an experiment recorded in Table V.

TABLE IV

Effect of Strong Immune Serum Added to Cells before and after the Addition of Virus

	Degree of consolidation of mouse lung			
	0	0	0	0
A. Immune serum 1 hr. before the virus	0	0	0	0
B. Normal serum 1 hr. before the virus	d++++	d++++	++++	++++
C. Virus 1 hr. before immune serum	0	0	0	0
D. Virus 1 hr. before normal serum	d++++	d++++	d++++	++++

TABLE V

Effect of Strong Immune Serum When Incubated with Virus for 15 Minutes before Addition of Cells

Titration of virus in cells and in washed ultracentrifuge sediment of culture supernatant fluid.

	Concentration in terms of original volume				
	10 ¹	Undiluted	10 ⁻¹	10 ⁻²	10 ⁻³
A. Sediment of culture supernatant					
Immune serum	0	±	0	0	
	0	±	0	0	
	0	+	0	0	
	0	0	0	0	
	0	0	0	0	
Normal serum	d++++	d++++	d++++	d++++	
	d++++	d++++	++++	++++	
	d++++	d++++	+++	++	
	d++++	d++++	+++	0	
	+	d++++	+++	+++	
B. Washed cells					
Immune serum		0	0	0	0
		0	0	0	0
		0	0	0	0
		0	0	0	0
		0	0	0	0
Normal serum		d++++	++++	Died	±
		d++++	++++	+++	0
		d++++	++++	+++	+
		++++	+++	++	++
		++++	+++	++	±

PR8 strain of culture virus (0.5 cc.) in 4.0 cc. of Tyrode solution, was incubated with 0.5 cc. of serum for 15 minutes. Then to each flask was added 0.2 cc. of minced 12 day chick embryo and the whole again incubated at 37°C. After 24 hours the cells were separated from the supernatant fluid by centrifugation, washed twice in Locke's solution, ground with sand in meat infusion broth, and the surviving virus titrated in mice. The supernatant fluids were centrifuged in an 8 inch rotor at 14,000 R.P.M. for 3 hours. The sediment was washed once in broth and tests for active virus in the sediment were made by the intranasal inoculation of the material into mice.

After exposure to immune serum, no virus was detected in the cell emulsion or to any significant extent in the sediment obtained from the supernatant fluid.

Experiments such as those already recorded are open to the criticism that the effect may be due to an action of the immune serum which prevents the virus from entering the cells, thus depriving the virus of a protective medium in the cells and subjecting it, therefore, to the destructive effect of a temperature of 37°C. Consequently, it seemed desirable to determine whether the effect of the immune serum can be exerted in the absence of cells and without prolonged incubation. To test this possibility, culture virus was incubated with serum at 37°C. for 30 to 40 minutes, the virus separated and washed free of excess serum, and the washed virus inoculated into mice. The following protocol gives the plan of such an experiment.

- | | |
|----------|---------------------------------|
| Flask A. | 2.0 cc. PR8 culture virus |
| | 0.5 cc. PR8 immune ferret serum |
| Flask B. | 2.0 cc. PR8 culture virus |
| | 0.5 cc. normal ferret serum |
| Flask C. | 2.0 cc. Tyrode solution |
| | 0.5 cc. PR8 immune ferret serum |
| Flask D. | 2.0 cc. PR8 culture virus |
| | 0.5 cc. normal ferret serum |

All four flasks were incubated for 35 minutes, after which the contents of each were centrifuged in the 8 inch rotor at 14,000 R.P.M. for 3½ hours. The supernatant fluids were discarded and the sediments washed twice in meat infusion broth (pH 7.8), and then suspended in 4.0 cc. of Tyrode solution, the sediments of C and D being combined to serve as a control for the possible sedimentation of the immune bodies of the serum. A portion of each of the three suspensions was diluted and the virus content titrated in mice. The remainder (3.0 cc.) of each was added to a 50 cc. Erlenmeyer flask containing 0.2 cc. of minced 12 day chick

embryo in 1.0 cc. of Tyrode solution. After 48 hours at 37°C., the cells from each of these cultures were ground with sand in their own supernatant fluids and the resultant suspensions tested for virus by mouse inoculation.

The results are given in Table VI. Such experiments seem unequivocal and indicate clearly that after 30 minutes' incubation at 37°C., immune serum affects the virus in such a way as to render it non-infectious for mice and to prevent its multiplication in the presence of

TABLE VI

Inactivation of Virus after Incubation with Strong Immune Serum for 30 Minutes in Absence of Cells

Dilution	Immune serum	Normal serum			Virus and sediment of immune serum		
A. Titration of virus in the twice washed ultracentrifuge sediment							
Undiluted	0 0 0	d++++	d++++	d++++	d++++	d++++	d++++
10 ⁻¹	0 0 0	d++++	d++++	d++++	d++++	d++++	d++++
10 ⁻²	0 0 0	++++	++	++	++	+++	++++
B. Titration of virus in 48 hr. tissue culture of the washed ultracentrifuge sediment							
Undiluted	0	d++++			d++++		
	0	d++++			d++++		
	0	d++++			d++++		
	0	d++++			d++++		
10 ⁻¹	0	d++++			d++++		
	0	d++++			d++++		
	0	d++++			d++++		
	0	++++			d++++		

susceptible cells. Normal serum or the sediment from immune serum was without effect.*

DISCUSSION

Most of the studies concerned with the question of union between antibody and virus *in vitro* have been carried out by means of the dilu-

* Further experiments have been completed in which it was found that the washed sediment obtained after centrifugation of the immune serum-virus mixture did not inactivate small amounts of added virus.

tion phenomenon in neutral or subneutral mixtures. Nevertheless, through all of them runs the suggestion that some reduction of virus titer occurs if the mixtures are incubated for 1 hour or more. This effect was more pronounced when immune serum was present in excess, frequently resulting in a failure to obtain reactivation. Sabin (8, 16), using high speed centrifugation for separation of vaccinia and pseudorabies viruses from serum-virus mixtures, has extended the findings obtained with other technics and has concluded, as have other workers, that no union between virus and protective substances occurs *in vitro* and that prevention of infection of susceptible cells is more closely related to fixation of immune substances by the cell.

The experiments reported were carried out with a strain of human influenza virus fully adapted to maintenance in tissue culture medium and with centrifugation at 14,000 R.P.M. in an air-driven high speed centrifuge for recovery of virus from the liquid portions of the medium.

The results of the present studies with the virus of human influenza have shown clearly that following incubation in the presence of sufficient antibody, virus is inactivated so that it is no longer detectable in sediments of immune serum-virus mixtures even after repeated washings and centrifugation. Under similar conditions in the presence of normal serum, virus so treated is fully infectious for mice. Furthermore, in the presence of cells, the virus is rendered inactive by a potent immune serum, no matter whether the cells are exposed first to virus or immune serum. These results cannot well be interpreted as due to an action of the immune substances on the tissue cells, since cells treated with immune serum and subsequently washed, adequately support the multiplication of the virus. They indicate rather that a pronounced effect of immune serum is exerted directly upon the virus. Procedures such as high speed centrifugation, which readily throws out the virus from normal serum-virus mixtures, fail to disrupt any association which may have occurred between immune substances and virus. Whether cells are required for the final disposal of the inactivated virus cannot be stated upon the basis of the present experiments. If cells are essential, it would appear that their function is to complete the destruction of the virus rendered inactive by the immune substances of the serum.

SUMMARY

Studies have been conducted on the effect of immune serum upon a strain of human influenza virus (PR8) grown in chick embryo tissue culture medium. The results have demonstrated (a) that when cells are exposed to the action of immune serum of high titer and subsequently washed freely, these cells support the growth of virus as well as cells treated with normal serum; (b) that, in agreement with the results of other workers, when virus is added to cell suspensions before the addition of immune serum of low titer, virus survives in the cells; (c) that when mixtures of immune serum of low titer and virus are added to cells, there is little evidence of survival or multiplication of the virus. Furthermore, when immune serum of high titer is used the virus is inactivated regardless of whether the cells are first exposed to virus or immune serum. Finally, virus mixed with a strong immune serum is inactivated in the absence of cells, as shown by the fact that centrifugation at high speeds of such serum-virus mixtures yields no active virus, whereas normal serum-virus mixtures yield fully active virus.

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THE AUTOLYTIC SYSTEM OF PNEUMOCOCCI

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Pneumococcus cells are extremely fragile; this is evidenced by the rapid autolysis which these organisms undergo both in culture media and in saline suspensions, by their solubility in bile, and by the ease with which they break up during repeated freezing and thawing. The possibility of obtaining pneumococci in "solution" has been considered a great advantage in the study of their antigenic structure, since it apparently offered a technique for the separation of the different cellular antigens. It is debatable, however, whether the methods which cause pneumococci to go into solution do not act by breaking down, chemically, some of the insoluble structures of the cell. Suggestive of this possibility are the following observations.

Whereas the immunization of rabbits (by intravenous route) with heat-killed cells of virulent pneumococci may bring about the production of precipitins directed against the type specific capsular polysaccharides of the strains used as antigens, these particular antibodies are not demonstrable when, instead of heat-killed cells, one uses for immunization, bacterial suspensions which have been allowed to undergo autolysis, or have been put in solution by bile, or by freezing and thawing (1). In fact, there are no convincing reports in the literature of the successful production of type specific precipitins directed against the capsular polysaccharide, by the intravenous immunization of rabbits with filtered soluble extracts of pneumococci. When one considers the paramount importance of this specific antibody in determining protection against pneumococcus infections, it becomes urgent to inquire into the possible mechanisms involved in determining the effectiveness of the proper antigen. As will appear from subsequent studies, the loss of specific antigenicity suffered by virulent pneumococci in the course of autolysis can be traced to the action of the cellular enzymes. On the other hand, the discovery

that active autolysates of pneumococci can cause the dissolution of heat-killed pneumococci (2) has made available a technique for the study of the phenomenon of autolysis itself. It seems justified, therefore, to devote the present paper to an analysis of some changes produced by sterile preparations of the autolytic enzymes of pneumococci upon heat-killed cells of the same bacterial species.

EXPERIMENTAL

Pneumococci were grown in peptone-beef heart infusion broth; the cells were collected by centrifugalization of active cultures which had been incubated at 37° for 6 hours.

1. *Bacteriolytic Enzyme*.—The autolysates were prepared by resuspending the cells in an amount of physiological saline corresponding to one-fifteenth the volume of the original culture. This suspension was incubated for 24 hours at 37°C. in the presence of an excess of toluol. The toluol, and the pellicle of fatty material adherent to it, was then discarded and the autolysate, consisting of Gram-negative detritus, was used as a crude preparation of bacteriolytic enzyme.

2. *Heat-Killed Cells*.—Young cells of pneumococci were resuspended in a very small amount of distilled water and the concentrated suspension rapidly added to a larger volume of distilled water at 75°C.; the temperature was maintained at this level for 20 minutes. This process of "flash" heating was selected because it minimizes the chances for alterations due to enzymatic action. The volume of fluid was so arranged that the final concentration of cells in the bacterial suspension was fifteen times greater than in the original culture.

The Effect of Autolysates upon Heat-Killed Pneumococci.—Avery and Cullen obtained from living pneumococci a heat-labile agent which caused rapid lysis of heat-killed cells of the same bacterial species, disintegrating the formed elements into a mass of Gram-negative detritus (2). This agent, the so called bacteriolytic enzyme, was found to be present in all pneumococci and to be effective against R or S variants of the cell, irrespective of type derivation. Similar findings were reported by Wollman (3). A quantitative estimation of the activity of the bacteriolytic enzyme of *Pneumococcus* is presented in the following experiment.

Experiment 1.—An active autolysate prepared from an R culture derived from Type II *Pneumococcus* was tested against heat-killed cells of the same R strain, and of an S strain of Type I. To a series of tubes each containing 0.5 cc. of the suspension of heat-killed cells and an equal volume of M/20 phosphate buffer (pH 6.5), the active autolysate was added in amounts varying from 0.5 cc. to

0.005 cc. as indicated in Table I. The mixtures were incubated at 37°C. for 18 hours in the presence of chloroform. At the end of the incubation period, films made of each of the preparations were stained by the Gram technique and the changes in morphological appearance and the staining properties of the cells were noted. At the same time, gross estimations of the degree of turbidity of the cell suspensions were also recorded (Table I).

The results recorded in Table I indicate that: (a) The bacteriolytic enzyme prepared from an R culture is effective against heat-killed cells of the same strain and also against those of encapsulated Type I pneumococci. However, it is possible that a more accurate titration

TABLE I

The Effect of Different Amounts of Active Autolysate upon Heat-Killed Pneumococci

Amount of autolysate II R	Suspensions of heat-killed cells		Microscopic appearance	Turbidity
	II R	IS		
cc.	cc.	cc.		
0.5	0.5	0	Gram-negative detritus	+
0.05	0.5	0	" cocci	+++
0.005	0.5	0	" "	++++
0.0005	0.5	0	Gram-positive "	++++
0	0.5	0	" "	++++
0.5	0	0.5	Gram-negative detritus	+
0.05	0	0.5	" cocci	+++
0.005	0	0.5	" "	++++
0.0005	0	0.5	Gram-positive cocci	++++
0	0	0.5	" "	++++

++++ indicates original turbidity of the cell suspension.

+++ , + indicate clearing of the cell suspension.

of enzyme and substrate might reveal quantitative differences between different strains. (b) When a large amount of bacteriolytic enzyme is used, the Gram-positive cocci are disintegrated into Gram-negative detritus; this change is accompanied by a marked clearing of the cell suspension. The form of dissolution thus induced is comparable to that which occurs during spontaneous autolysis. (c) With a smaller amount of bacteriolytic enzyme, the heat-killed cells become Gram-negative but still retain their characteristic morphology and there is no appreciable clearing of the cell suspension. (d) The agent which is responsible for the change from Gram-positive to Gram-negative is

present in great excess in the pneumococcus cell. In Experiment 1, 0.005 cc. of autolysate was sufficient to render Gram-negative the cells from 0.5 cc. of an equivalent bacterial suspension. In other words, each pneumococcus cell contains enough enzyme to render Gram-negative 100 heat-killed pneumococci (18 hours incubation at 37°C.).

Methods of Preparation of the Bacteriolytic Enzyme.—The ferment preparation used in the preceding experiment was an active autolysate containing the major part of the cell detritus. Attempts were made to purify the specific enzyme responsible for the change in the Gram reaction of the test cocci. This, however, proved a difficult task, due to the small amount of source material available and because the enzyme rapidly loses its activity during the manipulative procedures incident to its purification. Account was taken of the fact that the enzyme is readily adsorbed on the cellular detritus present in the autolysate especially at slightly acid reactions. If, for instance, an autolysate (which is normally slightly acid) is centrifuged at high speed, most of the activity is found in the deposit, whereas the supernatant is almost inactive. The following procedure was adopted for the preparation of a soluble enzyme.

1. The autolysate was adjusted to pH 4.7–5.0 with dilute acetic acid; after centrifugalization, the deposit washed with saline was found to contain the active principle.

2. The washed material was resuspended in $M/20$ phosphate buffer at pH 7.5, and after standing for 24 hours in the ice box, the suspension was centrifugalized and the supernatant fluid filtered through a Berkefeld candle (V) which had been washed with phosphate buffer solution at pH 8.0.

The filtrate was found to contain a large fraction of the activity of the original material. This Berkefeld filtrate can be concentrated by desiccation in the frozen condition and can be preserved in an active soluble form in a vacuum desiccator.

A certain degree of further purification may be achieved by precipitation with flavianic acid according to a method used for the preparation of the growth hormone of the anterior hypophysis (4) and of lysozyme (5). A solution of the active material is acidified with sulfuric acid (in the cold) until maximum precipitation is obtained (about pH 5.5). The inactive precipitate is discarded. The acidified

supernatant is then treated with sodium flavianate which precipitates all the active principle. This precipitate, carefully put back in solution by the addition of sodium hydroxide, is again precipitated with sulfuric acid, centrifuged, and finally brought into solution at neutral pH. When an effort was made to remove the flavianic acid from the precipitate by treatment with ammoniacal alcohol, 80 per cent of the activity was lost even when the extraction was carried out in the cold.¹

Certain Properties of the Bacteriolytic Enzyme.—The optimal range for the action of the bacteriolytic enzyme on heat-killed pneumococci is between pH 8.0 and pH 6.5; some slight action may still be detected at pH 5.5 and pH 8.5.

The enzyme preparation is unstable except when kept in the desiccated condition. It is completely inactivated in 10 minutes at 60°C. At 37°C. it remains stable for several days at pH 6.5 but loses its activity in a few hours at pH 5.0 and pH 8.0.

The enzyme is rapidly digested and inactivated by papain (activated with thioglycollic acid) at pH 7.0 and by trypsin at pH 8.0. It gradually loses activity by prolonged contact with bile, bile acids, and many fatty acids, both saturated and unsaturated; many antiseptics (such as formol and phenol) also inhibit the enzyme although ether, toluol, or chloroform are without effect. In view of these facts, a quantitative recovery of the enzyme cannot be expected when pneumococci are put in solution by bile; if an antiseptic is required, toluol or chloroform are to be given preference.

Neill and Avery (6) had already observed that the enzyme is destroyed by oxidation, especially when pneumococcus extracts are maintained under conditions which favor the formation of hydrogen peroxide. Neill and Fleming (7) found later that this oxidative inactivation may be reversed under certain conditions, by treating the inactive enzyme with reducing substances. The enzyme is also destroyed by a large excess of iodine. However, when smaller concentrations of iodine are used the inactivation is reversible. This phenomenon is illustrated in Experiment 2.

¹ The author is greatly indebted to Dr. Karl Meyer of the Ophthalmology Department of the Presbyterian Hospital, New York, for carrying out the purification of the enzyme by the flavianic acid treatment.

Experiment 2.—1 mg. of bacteriolytic enzyme (prepared from R cells derived from Type II Pneumococcus) was dissolved in 2 cc. of M/20 buffer pH 7.0. The enzyme solution was divided equally into four tubes; 0.5 cc. of M/75 iodine was added to two of these tubes (tubes 1 and 3), and 0.5 cc. of distilled water to the others (tubes 2 and 4). The solutions were then incubated for 75 minutes at room temperature.

At the end of this period, the iodine in tube 3 was reduced by the addition of 0.1 cc. of 0.3 per cent sodium thioglycollate; the bacteriolytic activity of the four preparations was then tested by the addition to each of 3 mg. of heat-killed pneumococci (Type I). The enzyme-substrate mixtures were incubated at 37°C. for 18 hours in the presence of chloroform and the activity of the enzyme estimated by Gram stains (Table II).

The results presented in Table II indicate that in the presence of iodine, the bacteriolytic enzyme loses the capacity to render Gram-

TABLE II
The Reversibility of Iodine Inactivation of the Bacteriolytic Enzyme by Thioglycollic Acid

Tube	Enzyme	M/75 iodine		0.3 per cent thioglycollic acid	Heat-killed bacteria		Gram stain
	cc.	cc.		cc.	mg.		
1	0.5	0.5	75 min. at room temperature	0	3	18 hrs. incubation at 37°C.	Gram-positive
2	0.5	0		0	3		Gram-negative
3	0.5	0.5		0.1	3		"
4	0.5	0		0.1	3		"

negative heat-killed pneumococci (tube 1); the bacteriolytic activity is restored, however, by the mere addition of thioglycollic acid to the iodine-inactivated solution, even though the iodine has been allowed to act for 75 minutes before the reducing agent is added (tube 3).

Observations on the Gram-Positive Structure of Pneumococci.—It was shown in Experiment 1 that it is possible, by the addition of small amounts of the bacteriolytic enzyme, to change heat-killed pneumococci into Gram-negative forms without at the same time causing any actual disintegration or lysis of the cells; it would be of the greatest interest to discover the nature of that structure of the cell which determines its reaction to the Gram stain. The following experiment is a contribution to this problem.

Experiment 3.—Heat-killed pneumococci (Type I) were washed twice with physiological saline and desiccated in the frozen condition. 100 mg. of the dry cells were resuspended in 20 cc. of M/20 borate buffer pH 8.5. To 10.0 cc. of this cell suspension was added 1 mg. of crystalline trypsin (recrystallized four times),² the remaining 10.0 cc. were kept as control. Both samples were incubated for 6 hours at 37°C., centrifuged, washed repeatedly with alcohol and ether at room temperature, then dried and weighed. The control bacteria were found to weigh 47.3 mg., whereas the sample treated with trypsin weighed only 12.1 mg.

This experiment was repeated on several occasions, with pneumococci of Types I and III. In all cases following the trypsin treatment there was a loss of weight corresponding to about 75 per cent of the original material. No appreciable change occurred when the treated cells were again subjected to tryptic digestion. The digestion was less complete with chymotrypsin.

On three occasions, the trypsin-digested cells (Type I) were analyzed for total nitrogen by the microKjeldahl method and found to contain from 7.57 to 8.43 per cent total nitrogen. The tryptic digestion was accompanied by a marked clearing of the preparation. However, this digested cell suspension revealed the presence of Gram-positive cocci somewhat smaller in size than the control organisms but comparable in numbers and morphological appearance.

The digested cocci were resuspended in phosphate buffer solution at pH 6.5, in which they retained their morphological and staining characteristics. If now a sufficient amount of the bacteriolytic enzyme was added to this suspension, the cells were changed into Gram-negative detritus, although there was no appreciable decrease of the turbidity of the suspension.

The results of Experiment 3 seem to indicate that the Gram-positive reaction is associated with the existence of a structure which still persists, after trypsin digestion has reduced the heat-killed pneumococcus cells to bodies which weigh only 25 per cent of the original weight of the cell and contain only 8 per cent nitrogen. Although the bacteriolytic enzyme can attack this structure, it cannot dissolve it completely, but leaves in suspension an amorphous insoluble Gram-negative residue.

The Inhibition of the Bacteriolytic Enzyme by Certain Amino Polysaccharide Acids.—It has been shown elsewhere that a preparation of the bacteriolytic enzyme has the property of liberating reducing sugars from certain acetyl amino glucose glucuronides prepared from the vitreous humor, from the umbilical chord, and from cultures of hemolytic streptococci of group A (8). If one and the same enzyme

² The crystalline trypsin and chymotrypsin were kindly supplied by Dr. J. H. Northrop.

is responsible for the action on these amino polysaccharide acids and on heat-killed pneumococci, one might expect that the former substances would inhibit the action of the enzyme upon the pneumococcus substrate. This is established in the following experiment.

TABLE III

The Inhibition of the Bacteriolytic Enzyme by Certain Amino Polysaccharide Acids

Enzyme	Inhibiting substance in 2 cc. pH 6.0 buffer		Amount of enzyme inhibitor mixture used	Test material		No. of Gram-negative cocci
cc.			cc.			
0.1	0	7 hrs. incubation at 37°C.	0.01	1 mg. cocci + 1 mg. vitreous polysaccharide	16 hrs. incubation at 37°C.	All
			0.01	1 mg. cocci + 1 mg. umbilical polysaccharide		"
0.1	5 mg. vitreous humor polysaccharide		0.1	1 mg. cocci		None
			0.02	1 " "		"
0.1	2 mg. vitreous humor polysaccharide		0.1	1 " "		All
			0.02	1 " "		50 per cent
0.1	5 mg. umbilical chord polysaccharide		0.2	1 " "		None

Experiment 4.—Mixtures of the bacteriolytic enzyme (prepared from an R Pneumococcus derived from Type II) and of the amino polysaccharide acids were prepared in the following proportions.³

- (c) 0.1 cc. enzyme + 2 cc. phosphate buffer M/20 pH 6.0.
- (b) " " " + 5 mg. vitreous polysaccharide in 2 cc. buffer pH 6.0.
- (c) " " " + 2 mg. " " " " " " " " " "
- (d) " " " + 5 mg. umbilical " " " " " " " " " "

The mixtures were incubated at 37°C. for 7 hours; at the end of this period the bacteriolytic activity of the preparations was tested against 1 mg. dried pneumococci resuspended in 2 cc. of M/20 phosphate buffer pH 7.0. The enzyme-substrate

³ The samples of amino polysaccharide acids were kindly supplied by Dr. Karl Meyer.

mixtures were incubated at 37°C. for 18 hours and the amount of lysis determined by Gram stains. Table III presents the results of the experiment.

The results given in Table III show that the enzyme solution was of such concentration that 0.01 cc. was sufficient to render all the pneumococci Gram-negative. When the enzyme was incubated with 5 mg. of the amino polysaccharide acids previous to the addition of the pneumococci, all the bacterial cells remained Gram-positive. With only 2 mg. of amino polysaccharide the lytic activity was only slightly reduced.

In other words, when the bacteriolytic enzyme is incubated for 7 hours with a sufficient amount of the amino polysaccharide acids, it loses thereby its ability to change the pneumococci from Gram-positive to Gram-negative; the polysaccharide acids, however, do not exert an inhibiting effect by their mere presence, since the bacteria are readily attacked by the bacteriolytic enzyme when inhibitor and bacteria are added simultaneously to the enzyme solution. It appears possible, therefore, that the bacteriolytic agent becomes fixed during the decomposition of the acetyl amino polysaccharide and subsequently is no longer available to attack the bacterial cells.

DISCUSSION

The rapidity with which pneumococci undergo autolysis is one of their significant characteristics. This property is probably related to the presence in the cell of the lytic system which Avery and Cullen (2) have termed the bacteriolytic enzyme. It is likely that the autolytic system is composed of a group of different enzymes, each one of them acting upon a different component of the cellular structure. When used in large amounts, the bacteriolytic system changes Gram-positive heat-killed pneumococci into amorphous Gram-negative detritus. However, the enzyme solution can be used under such conditions that the bacterial cells become Gram-negative, but retain their characteristic morphology and that there is no clearing of the bacterial suspension; in other words, the change in staining properties need not be associated with an actual lysis of the cell.

What is the structure upon which the enzyme acts to render the pneumococci Gram-negative? We know that it is resistant to proteolytic enzymes (pepsin, papain, trypsin) and that it is still present

after trypsin digestion has reduced the cell to a body which contains only 8 per cent nitrogen (Experiment 3). Moreover it has been found that the same enzyme preparation which attacks this structure is also capable of liberating reducing sugars from certain acetyl amino glucose glucuronides (8). The agents responsible for both types of action are heat-labile, have the same pH optimum, are precipitable by flavianic acid, are reversibly inactivated by iodine, and finally, the action on pneumococci is inhibited by previous incubation of the ferment solution with sufficient amounts of the amino polysaccharide. These facts suggest that the same component of the bacteriolytic system which hydrolyzes the amino polysaccharide acids also renders Gram-negative the heat-killed pneumococci. This question, however, will not be finally answered until one has identified in pneumococci, the specific substrate which, when present as an organized structure, determines the Gram-positive reaction of this group of bacteria.

SUMMARY

1. Living pneumococcus cells contain a group of enzymes, the bacteriolytic system, capable of causing the lysis of heat-killed pneumococci (R and S variants irrespective of type derivation). This lysis expresses itself by a loss of the Gram staining reaction, a disintegration of the cell body, and a clearing of the bacterial suspension.

2. Under certain conditions of treatment with the bacteriolytic complex, it is possible to render the cocci Gram-negative without changing their characteristic morphology, or causing any appreciable clearing of the cell suspension.

3. The enzyme responsible for this change has been partially purified, and some of its properties described.

4. The cellular structure which is responsible for the Gram-positive reaction of pneumococci is resistant to proteolytic enzymes, and is still present when tryptic digestion has reduced the heat-killed cell to a body which has lost 75 per cent of its original weight, and contains only 8 per cent nitrogen.

5. The same enzyme preparation which attacks pneumococci is also capable of liberating reducing sugars from some acetyl amino glucose glucuronides of animal and bacterial origin. The possibility is con-

sidered, and discussed, that one and the same enzyme in the autolytic complex is capable of attacking both types of substrates.

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CHEMICAL STUDIES ON BACTERIAL AGGLUTINATION

III. A REACTION MECHANISM AND A QUANTITATIVE THEORY*

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Recent and even current articles on specific bacterial agglutination reveal wide differences of opinion regarding the mechanism of the reaction and the factors influencing its course. Since much of this divergence appeared traceable to the essentially qualitative and relative nature of the methods used for the estimation of agglutinin titer, it was decided to attempt the assembly of more precise data on agglutination than had hitherto been available. As a first step an absolute, quantitative method was developed for the micro estimation of certain agglutinins (1), in which these are measured as the amount of antibody nitrogen actually combined with a given volume of bacterial suspension and in which an accuracy of 0.01 to 0.02 mg. of nitrogen is easily attainable. With this method and with the absolute, quantitative methods previously developed for the estimation of precipitins (2-4), it was possible to demonstrate the identity of agglutinin and precipitin in absorbed antisera containing only antibody to the type specific polysaccharide of Type I pneumococcus (5). It has now been possible to extend the analogy and to account quantitatively for this instance of specific bacterial agglutination by a purely chemical theory, much as had been accomplished in the case of the precipitin reaction (6).

EXPERIMENTAL

Heat-killed suspensions of Type I S (Dawson M) pneumococci were prepared as described in (1). It was found that on standing for some time appreciable amounts of polysaccharide appeared in the supernatants of even carefully washed

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suspensions, just as Craigie and Wishart (7) found antigen to leach out slowly from washed suspensions of the elementary bodies of vaccinia virus. In order to avoid confusion of the agglutinin reaction under investigation with a simultaneously occurring precipitin reaction, the suspensions were freshly centrifuged and washed once with saline before use. After this treatment only insignificant traces of specific polysaccharide remained in the supernatant.

Type I antipneumococcus horse sera H 701, H 702, and a Felton antibody solution B 78 were completely freed from group specific antibody by repeated absorption with I R (Dawson S) pneumococci as described in (5). Horse serum H 79 was absorbed with C substance (8) and protein prepared from a Type I R strain. Felton antibody solution B 75 was described in (1). The pH of antibody solution B 78 was 6.96, that of serum 701 was 7.34.

Estimation of the amount of S I¹ in the pneumococci was carried out as in (5) by dissolving a measured volume of the suspension in alkali, neutralizing after 72 hours at 37°C., and making up to a volume calculated to yield 0.9 per cent salt

TABLE I

Effect of Volume and Final Concentration of Antibody on the Amount of Antibody N in Agglutinated Pneumococci

Volume	Antibody N removed by 0.20 mg. bacterial N	Antibody N concentration in supernatant
ml.	mg.	mg. per ml.
4.0	0.33	0.15
8.0	0.34	0.074
12.0	0.33	0.050

concentration. Aliquot portions of this solution were analyzed according to (4) by means of an antibody solution calibrated with alkali-treated S I.

Agglutinin determinations were carried out by addition of accurately measured volumes of washed bacterial suspension to measured volumes of serum or antibody solution and estimation of the increase in nitrogen content. Heat-killed suspensions of Pn I were used. All determinations were run in duplicate unless otherwise stated and tubes were covered with tight fitting rubber caps.

Data on the effect of volume on the agglutination reaction in the region of excess antigen were included in (1). Additional data in the region of excess antibody are given in Table I. It will be seen that the amount of antibody nitrogen taken out by the bacteria after 48 hours at 0°, with occasional stirring, is independent of the volume just as in the precipitin reaction (6). Thus the agglutinin nitrogen

¹ This designation is used for the specific polysaccharide of Type I pneumococcus. Pneumococcus I suspensions are referred to as Pn I.

removed is independent of the concentration of antibody nitrogen in the supernatant.

In Table II are given data in the region of excess antibody on the effect of varying lengths of exposure of the Pn I suspensions to antibody. Suspension and serum were mixed in tubes and allowed to stand with occasional stirring. Increasing amounts of antibody nitrogen were taken up with increasing lengths of time.

The extremely slow rate under these conditions at 0° suggested the possibility of measuring the velocity of agglutination. 15 ml. of Pn I and 3 ml. of antibody

TABLE II

Effect of Time of Standing on Antibody N Taken up by Pn I at Various Temperatures

Time	Felton antibody solution B 75	Serum H 79			
		0°	25°	37°	55°*
hrs.	mg.	mg.	mg.	mg.	mg.
1					0.46†
2				0.37	
6					0.53
19	0.33	0.27	0.36	0.44	
24					0.58
43			0.39		
48				0.44	0.58
72	0.40				
days					
5			0.43		
10				0.52	
12		0.34			

* Control tubes containing serum alone tended to gel at this temperature unless diluted with saline.

† One determination.

solution B 78 were added to 32 ml. of saline and the mixture was stirred mechanically in an ice bath. The temperature remained constant at +0.5°C. 5 ml. samples were withdrawn at varying intervals and pipetted into 5 ml. of cold saline and centrifuged at about 3000 R.P.M. in a refrigerated centrifuge.² The precipitates were washed and analyzed in the usual manner. It will be seen from Table III that with efficient stirring the reaction is an extremely rapid one, being more than 80 per cent complete in 5 minutes. Maximum combination of antibody requires a considerably longer period. Even when this is attained the aggregates

² Manufactured by the International Equipment Company, Boston.

formed are much smaller than in the incomplete reaction under the usual conditions.

In accordance with the above experiments it was found possible to use the usual arrangement of tubes and take out the maximum antibody nitrogen for any given set of conditions if the mixtures were shaken on a shaking machine oscillating with sufficient rapidity to keep the Pn I in suspension. A machine of the type² adopted for streptolysin determinations proved adequate. Shaking at 37° was carried out in an incubator, and at 0° the tube racks were placed in a box, packed with crushed ice, and shaken, the ice being replenished as often as necessary. Table IV shows the result of shaking for varying lengths of time. Values in parentheses were obtained by use of a mechanical stirrer instead of shaking. It will be seen that within the limit of error of the determinations stirring and shaking yielded identical results. Since the dilution in the stirred mixtures was more than two and one-half times as great, this confirms the finding (Table I) that antibody N removed is independent of the concentration. Two different lots

TABLE III
Velocity of Combination of Antibody and Pn I

Time	Antibody N removed	Time	Antibody N removed
<i>min.</i>	<i>mg.</i>	<i>min.</i>	<i>mg.</i>
5	0.12	60	0.15
15	0.14	90	0.16
25	0.14	120	0.16
40	0.14	150	0.16

Samples taken contained 0.25 mg. bacterial N.

of Pn I were used for the 37° and 0° experiments with B 78, but for sera H 701 and H 702 the same suspension was used for all determinations and results at different temperatures and salt concentrations are strictly comparable. It will be observed that in the region of excess antibody much more agglutinin N was taken out by the same amount of suspension at 37° than at 0°. This will be discussed below.

After determination of the time necessary to reach equilibrium, a series of tubes containing 1.0 ml. of serum or antibody solution and increasing amounts of freshly washed Pn I was set up in duplicate and shaken at the desired temperature until equilibrium was reached. The tubes were then centrifuged at the same temperature and the precipitates washed twice in the cold (1) and analyzed for nitrogen. Agglutinin N was found by subtracting the bacterial N. At the same time an accurately measured portion of Pn I was dissolved in alkali and the S content determined as described above. In the experiments in which the final salt concentration was to be 2 M, a volume of 3.85 M salt equal to the volume of serum and suspension used was first added. Table V shows the results obtained

TABLE IV
Antibody N Removed from Antipneumococcus I Solution and Sera by Pn I on Shaking for Varying Periods

Time	B 78				H 701				H 702			
	0.9 per cent NaCl		0.9 per cent (0.15 M) NaCl		2 M NaCl		0.9 per cent (0.15 M) NaCl		0.9 per cent (0.15 M) NaCl		2 M NaCl	
	37°	0°	37°	0°	37°	0°	37°	0°	37°	0°	37°	0°
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Int.	0.22	0.22										
1	0.23*	0.23	0.31	0.13	0.24*	0.15	0.27	0.16	0.25	0.17	0.19	0.17
1.5	0.23	0.26*	0.35	0.14	0.23 (0.22)	0.15	0.28	0.17	0.25	0.19	0.20 (0.19)	0.16
2			0.35 (0.32)	0.14 (0.13)		0.16	0.29† (0.25)	0.17 (0.21)	0.25 (0.23)	0.20 (0.19)		
3						0.16*						
4												
5												
6												
7.5												
Bacterial N used	0.35	0.34	0.12	0.09	0.11	0.09†	0.16	0.16	0.16	0.16	0.16	0.16

Values in parentheses obtained with mechanical stirring instead of shaking. Aliquot portions analyzed in triplicate.

* One determination only.

† A duplicate set of tubes, after 6 hours at 37°, was shaken 8 hours at 0°, yielding 0.32 mg. of antibody N.

‡ Bacterial N of the suspension in 0.9 per cent NaCl was used, owing to the difficulty of centrifuging non-agglutinated Pn I in 2 M NaCl.

Bacterial N	Equivalent S content	Total N precipitated	Antibody N precipitated	Ratio N:S in precipitate	Antibody N calculated from equation
mg.	mg.	mg.	mg.		mg.
0.122	0.017	0.255	0.17	7.1*	0.24
0.214	0.034	0.494	0.24	7.1	0.34
0.366	0.051	0.718	0.34	6.7	0.41
0.488	0.068	0.916	0.41	6.0	0.56
0.732	0.102	1.302	0.56	5.5	0.59
0.976	0.136	1.576†	0.59	4.3	0.61
1.220	0.170	1.840	0.61	3.6	0.60
1.464	0.204	2.076	0.60	2.9*	0.014
Serum, salt		0.014			
		mg. antibody N pptd. = 8.0 S - 26.9 S ²			
		S max. = 0.149 N max. = 0.594 calcd.			
		0.61 found			
		Serum H ⁺ 701, 0.15 M Salt, at 37°			
0.064	0.0165	0.254	0.18	10.9*	0.28
0.096	0.025	0.369	0.27	10.8	0.36
0.127	0.033	0.476	0.34	10.3	0.49
0.191	0.0495	0.686	0.49	9.9	0.59
0.255	0.066	0.868	0.61	9.2	0.72
0.382	0.099	1.060	0.67	6.8	
0.509	0.132	1.202	0.69	5.2	
0.637	0.165	1.340	0.70	4.2*	
Serum, salt		0.008			
		mg. antibody N pptd. = 12.5 S - 53.2 S ²			
		S max. = 0.1175 N max. = 0.734 calcd.			
		0.70 found			
		mg. antibody N pptd. = 5.7 S - 11.6 S ²			
		S max. = 0.246 N max. = 0.70 calcd.			
		0.69 found			
		H ⁺ 701, 0.15 M Salt, at 0°			
		0.187	0.12	7.3*	0.47
		0.290	0.19	7.6*	0.63
		0.376	0.25	7.6*	0.74
		0.534	0.34	6.9*	0.79
		0.722	0.47	7.1	
		1.030	0.65	6.6	
		1.244	0.74	5.6	
		1.414	0.78	4.7	
		0.000			
		mg. antibody N pptd. = 8.8 S - 24.5 S ²			
		S max. = 0.180 N max. = 0.79 calcd.			
		0.78 found			

	H 701, 2 M Salt, at 37°			H 701, 2 M Salt, at 0°		
	0.064	0.0165	0.222	0.16	9.7	0.15
Serum, salt	0.096	0.025	0.290	0.19	7.6	0.20
	0.127	0.033	0.354	0.23	7.0	0.24
	0.191	0.0495	0.454	0.26	5.3	0.28
	0.255	0.066	0.540	0.29	4.4	
	0.382	0.099	0.688	0.31	3.1*	
	0.509	0.132	0.828	0.32	2.4*	
	0.637	0.165	0.980	0.34	2.1*	
		0.000				
			mg. antibody N pptd. = 10.6 S - 100 S†			
			S max. = 0.053 N max. = 0.28 calcd.			0.34 found
For serum H 702 the equations were:						
0.15 M salt: At 37°, mg. antibody N pptd. = 6.9 S - 16.5 S†						
S max. = 0.209 N max. = 0.72 calcd.						
2 M salt: At 37°, mg. antibody N pptd. = 7.7 S - 44.8 S†						
S max. = 0.086 N max. = 0.33 calcd.						
0.74 found						
At 0°, mg. antibody N pptd. = 5.8 S - 11 S†						
S max. = 0.263 N max. = 0.76 calcd.						
0.83 found						
At 0°, mg. antibody N pptd. = 6.3 S - 20.7 S†						
S max. = 0.152 N max. = 0.48 calcd.						
0.53 found						

* Points not considered in calculating equation.

† One determination discarded.

‡ Only two points not at maximum ratio available for calculation.

in these experiments. The ratios given were calculated from the determinations of antibody N in the agglutinated Pn I and the S I content of the Pn I suspension used, although it is by no means certain that all of the S I in the suspension is capable of reacting. If it is assumed that the same proportion of the S I reacts at a given temperature throughout the entire reaction range, the observed N:S ratios would then be divided by the same factor and the character of the results would be unchanged. It will be seen from Table V that the ratios reach an upper limit with decreasing amounts of suspension. This was also determined by the

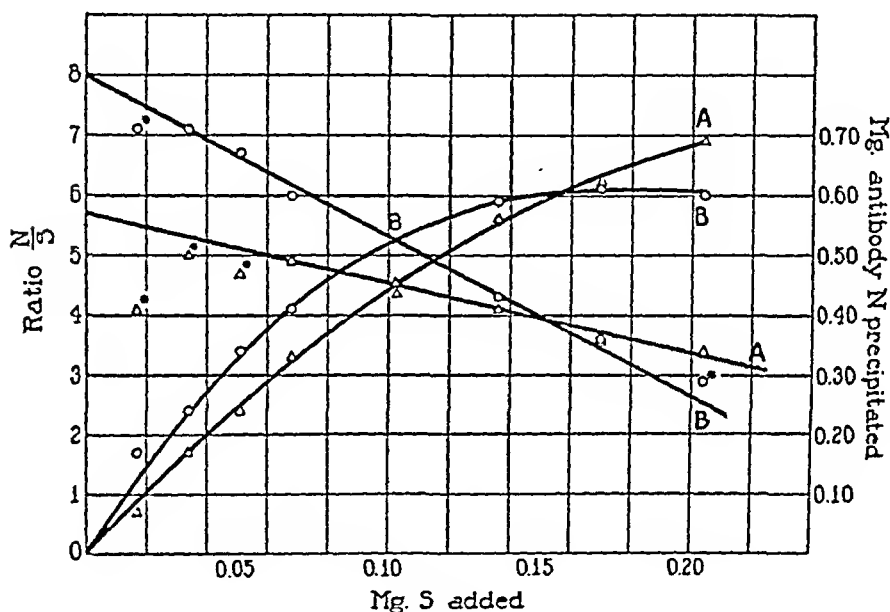


FIG. 1

addition of a constant amount of Pn I to increasing quantities of serum H 79 at 37°.

Volume of serum, ml.	0.5	1.0	1.5	2.0	2.5	3.0
Antibody N removed, mg..	0.47	0.53	0.60	0.63	0.62	0.63

The amount of antibody N removed by 0.26 mg. of bacterial N reached a definite limit as the volume of serum added was increased.

In the region in which a change of ratio is observed the equation

$$N = 2RS - \frac{R^2}{A} S^2$$

derived for the precipitin reaction (6) was found to hold. In Table V are given the equations calculated from the data at each temperature and salt concentration. The antibody N values found are also compared with those calculated from the above equation. Fig. 1 shows the curves obtained in the case of antibody solution B 78 at 0° (curve A) and at 37° (curve B) in 0.9 per cent saline by plotting antibody N in the agglutinated Pn I against the polysaccharide content of the Pn I. Lines satisfying the equation

$$\frac{N}{S} = 2R - \frac{R^2}{A} S$$

were obtained by plotting against S I the ratios of agglutinin N taken out to S I in the Pn I used (Fig. 1, lines A and B). The method of least squares was used in

TABLE VI
Comparison of Maximum Calculated and Found Antibody N:S I Ratios

Serum	Maximum N:S I ratio from equation	Maximum N:S I ratio found	Calculated ratio Found ratio
701, 0.15 M salt, 37°	12.5	10.0	1.25
" " " " 0°	8.8	7.4*	1.19
" 2 " " 37°	10.6	9.7	1.09
" " " " 0°	9.9	8.5	1.17
B 78, 0.15 " " 37°	8.0	7.1	1.13
" " " " 0°	5.7	4.6*	1.24
702, 0.15 " " 37°	6.9	5.4*	1.28
" " " " 0°	5.8	4.2*	1.38
" 2 " " 37°	7.7	6.7	1.15
" " " " 0°	6.3	5.6*	1.12

* Mean of maximum ratios determined experimentally.

every case to calculate the line best fitting the data. Points in the region of excess antigen, as well as those beyond the first point with maximum ratio, were not used in the calculations. These points are marked with asterisks in Table V and Fig. 1. The data obtained in 2 M salt solution are also in agreement with corresponding results for the precipitin reaction (9). Similar data were obtained with serum H 702 in 0.15 and 2 M salt solution at both temperatures.

A comparison of the maximum calculated and found ratios of antibody N to S is made in Table VI. It will be seen that the theoretical maximum ratio, 2 R, (i.e., the intercept of the line on the y axis) is approximately equal to 1.2 times the experimentally determined maximum ratio.

It will be noted from Table V and Fig. 1 that throughout the region of antibody excess a given amount of Pn I removes more antibody N at 37° than it does when set up at 0° with serum in the same proportions. The following experiment was

performed in an effort to explain this effect. Four tubes containing 1.0 ml. of Pn I suspension and 1.0 ml. of serum were shaken for 6 hours at 37°. One pair was then shaken at 0° for 8 hours. Agglutinin N was determined in both sets of tubes. It was found that more agglutinin N was removed by shaking at 37° and then at 0° than was taken out at 37° alone (see footnote, Table IV). The difference in N removed at 37° and at 0° is very markedly reduced in 2 M salt.

As in the precipitin reaction it was not found possible to take out at 0° antibody remaining after the maximum antibody had been taken out at 37° with excess Pn I.

In the region of excess antibody, the agglutinated bacteria are very easily and uniformly resuspended in saline. In the region of excess polysaccharide, as in the precipitin reaction, large clumps are formed which cannot be easily dispersed.

To determine whether or not Pn I, agglutinated in the region of excess antibody, is capable of combining with more Pn I, the following experiment was set up.

To each of a series of tubes were added 0.5 ml. of Pn I S suspension (0.19 mg. N) and 1.5 ml. of serum, a large excess. The agglutinated bacteria were allowed to stand in the ice box for 48 hours and were centrifuged and washed twice with saline. The second washing was set up with 0.25 ml. of fresh Pn I suspension and failed to show agglutination, indicating that all uncombined antibody had been washed out. The original agglutinated (sensitized) Pn I was then resuspended uniformly in saline, and 0.25 ml. portions of various pneumococcus suspensions or measured amounts of specific polysaccharide (S I) were added. The results are shown in Table VII.

Eagle, Smith, and Vickers (10) have found that the partial coupling of pneumococcus Type I antibody with diazotized sulfanilic acid prevented the antibody from precipitating S I although it still gave definite agglutination with Pn I. It was concluded that S I failed to combine with the azo antibody since addition of untreated Type I antipneumococcus serum gave a precipitate. However, agglutination would also fail to occur if the polysaccharide no longer combined with antibody, since it has been shown that Type I anticarbohydrate precipitin and agglutinin are identical (5). If, on the other hand, soluble compounds of S I and azo antibody were formed, such compounds should be as readily capable of reacting with unaltered antibody to form precipitates as is uncombined S I. It has long been known that polysaccharide and antibody can combine in proportions varying over a wide range (11). Eagle, Smith, and Vickers' experiment was accordingly repeated with additional controls.

6 ml. of Type I antipneumococcus horse serum were coupled with 6 ml. of diazotized sulfanilic acid as described in (10). 2 ml. of the resulting azo antibody, freed from excess diazosulfanilic acid, failed to precipitate 0.05 mg. of S I or its deacetylated degradation product, but agglutinated 2 ml. of Pn I suspension (0.2 mg. N per ml.). 1 ml. of Type I antiserum was added to the tubes containing S I, resulting in immediate precipitation. As an additional control, an egg albumin-anti-egg albumin precipitate was formed in 2 ml. of the azo antibody solution by addition of 0.34 mg. of egg albumin and 0.5 ml. of a potent anti-egg albumin serum. All tubes were centrifuged and washed in the cold with saline until the washings were colorless. The egg albumin-anti-egg albumin precipitate was white, while the precipitates in the other tubes were definitely yellow. S I had therefore combined specifically with the azo antibody, not only on the surface of Pn I, where agglutination occurred, but in solution as well.

DISCUSSION

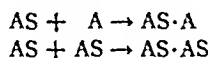
The mechanism of specific bacterial agglutination (reviewed in (12, 13, 14)) has been considered either as an adsorption process or as a precipitin reaction taking place at the cell surface (15). Although adsorption is now generally considered in its chemical aspects, Ivanovics (16) as late as 1935 chose to interpret bacterial agglutination as an example of physical adsorption. This conclusion is open to criticism on account of the large error of the experimental method used, the failure to eliminate simultaneously occurring precipitin reactions, and the complexity of the antigen-antibody system studied.

From the data given in Table I and reference 1 it is evident that a simple treatment according to classical chemical laws is as inadequate in accounting quantitatively for specific bacterial agglutination as is the Freundlich adsorption isotherm, since both depend on the concentrations of the reactants at equilibrium. As in the precipitin reaction (6) the composition of the agglutinated mass depends rather on the proportions in which the components are mixed (Table V).

In order that figures such as those given in Table V should be of significance it was necessary first to find the conditions under which the maximum amount of antibody nitrogen would be taken out by Pn I throughout the reaction range. It was observed that removal of antibody was too slow when tubes were mixed only at intervals (Table II), although this procedure was satisfactory in the precipitin reaction. This was due not to a slower rate of combination of polysaccharide and antibody in agglutination, but more probably to the fewer collisions between Pn I and antibody molecules. Mechanical stirring (Table

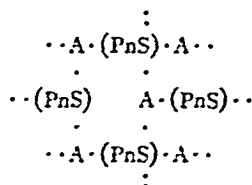
III) resulted in rapid attainment of maximum combination, as did also the use of a shaking machine (Table IV). A technique was thus available for a study of the agglutination reaction similar to that made of the precipitin reaction.

The data so obtained (Table V) indicate that equations of the same form as were valid for the precipitin reaction (6) serve also to describe quantitatively the agglutination reaction between Pn I and homologous type-specific anticarbohydrate. In the derivation of these equations it was assumed that S (polysaccharide) and A (antibody) first combine in a bimolecular reaction to yield AS, followed by the competing bimolecular reactions (in the region of excess antibody),



and that reactions of this type continue with increasing complexity until aggregates are formed which are large enough to separate from solution. In the derivation, also, it was found that volume factors cancelled, so that the end-result was independent of the final concentrations of the components. Since this condition is also shown experimentally to obtain in the agglutination reaction studied (Table I), and since a similar type of equation is found to apply to this reaction (Table V) as well as to specific precipitation, it would appear justifiable to consider this instance of specific bacterial agglutination as a precipitin reaction at the bacterial surface,³ the more so as the same substances enter into both reactions (5).

According to this mechanism the mass of agglutinated Pn I would consist of pneumococci held together by combination of S I on the pneumococcus surface with antibody molecules. This three-dimensional process might be represented two-dimensionally as follows:



³ In this discussion "bacterial surface" is defined as the portion of the Pn I cell to which reactive S I molecules are attached. This would not necessarily coincide with the external surface.

in which (PnS) represents pneumococcus with an unknown number of molecules of S at the reactive surface or surfaces. A somewhat similar qualitative representation of agglutination has been given by Marrack (14).

Agglutination of Pn I differs in at least three respects from the precipitin reaction. It will be noted from Table V that the antibody N:S I ratios reach a maximum in the region of excess antibody, beyond which more antibody cannot be forced into reaction. The value of 2 R given by the equation is usually about 1.2 times the experimentally determined maximum, as shown in Table VI. A possible explanation would be that antibody molecules, when present in large excess, are prevented by spatial exigencies from combining with all of the immunologically reactive S groupings available. It appears reasonable to assume that this crowding would occur at lower N:S I ratios at the Pn I surface than in the precipitin reaction, in which S I is in solution.

Another difference from the precipitin reaction is in the apparently lower combining ratios of the components. Since it is not known whether or not all of the S found in Pn I is available for reaction, as is assumed in calculating the ratios given, these values have only a relative significance. If, under a given set of conditions, the same fraction of S I reacts throughout the whole range, the entire set of ratios could be multiplied by a factor inversely proportional to the fraction reacting.

A third difference is that throughout the antibody excess range more antibody is taken out at 37° than at 0° (Table V and Fig. 1). An even larger amount of antibody N is removed by the same quantity of Pn I when the reaction is carried out first at 37° and then at 0° (footnote, Table IV). It would seem that a larger proportion of the S I present is capable of reacting at 37° than at 0°, but when this has once entered into reaction, continuation of the process at 0° takes place much as in the precipitin reaction, with more antibody removed at the lower temperature. Possibly the effect noted is due to swelling of the Pn I at 37°, resulting in the exposure of more of the S I content.

The differences between the precipitin and agglutinin reactions are thus seen to be mainly mechanical, rather than differences in principle, and to be conditioned by the spatial limitations of a chemical reaction taking place, in agglutination, at the bacterial surface. Since con-

siderable confusion would be caused by the adoption of conclusions of Eagle, Smith, and Vickers (10) indicating a fundamental difference between agglutination and precipitation, it is necessary to mention here that these conclusions are easily shown to be based on insufficient evidence. By the repetition of their experiments with the necessary controls (page 895) it has been shown that Type I pneumococcus antcarbohydrate which has been coupled with diazotized sulfanilic acid actually combines with S I, although it does not yield a precipitate. It is therefore not surprising, in view of the existing knowledge of the precipitin reaction and the demonstrated identity of antcarbohydrate precipitin and agglutinin (5) that agglutination of Pn I is effected by the same azo antibody which merely combines with S I in solution to give soluble compounds.

According to the new quantitative theory the entire process of specific bacterial agglutination may be most simply treated as a continuous progression of competing bimolecular chemical reactions. The theory makes no distinction between the initial combination of antigen with antibody and the subsequent aggregation, the latter being considered as a continuation of the process of combination of multivalent antigen with multivalent antibody until aggregates are formed which are large enough to flocculate.

While the presence of salts is necessary for flocculation, and their function is discussed in greater detail below, it is believed that the experiments recorded in Table VII indicate the essentially chemical nature of even the flocculation stage of specific bacterial agglutination. By interruption of the process of specific bacterial agglutination at a definite stage it was found possible to study the specificity of further particulation under constant conditions of salt concentration and to test predictions based on the theory.

Pn I cells, agglutinated with a considerable excess of antiserum, were washed with saline until the supernatant contained no agglutinin. The agglutinated (sensitized) Pn I cells were then evenly resuspended in saline and divided into several portions. According to the new quantitative theory, Pn I, agglutinated in the region of excess antibody, would still have available on the surface of the particles some of the specifically reactive groupings of the originally multivalent antibody. These particles, then, should be able to combine with S I on

the surface of freshly added unsensitized Pn I, and reagglutination should take place to form larger aggregates. It will readily be seen from Table VII that this prediction was verified, and that the effect is specific, since it is not given by Pn II or III, or by Pn I R (Dawson S) cells under identical conditions of salt concentration and, in the

TABLE VII

Addition of P. Pneumococcus or S I to Washed, Resuspended, Agglutinated Pn I S (M)

Material added	Result
Unsensitized Pn I S (M) suspension	Rapid reagglutination into large clumps, clear supernatant
" Pn I R suspension	No visible reaction
Pn I S supernatant*	" " "
Unsensitized Pn III S (M) suspension	" " "
" Pn II S (M) suspension	" " " †
1 mg. S I (Pn I specific polysaccharide)	" " "
0.10 mg. S I	Reagglutination‡
0.01 mg. S I	" †
0.001 mg. S I	Partial reagglutination
0.0001 mg. S I	" "
0.10 mg. S II	No visible reaction
0.9 per cent saline	" " "

Reagglutination failed to take place if sensitized Pn I and fresh Pn I were washed with 5 per cent glucose until supernatants no longer reacted for Cl^- . Addition of NaCl caused immediate flocking. The function of electrolyte is discussed in the text.

* Freshly washed, unsensitized Pn I S suspension was again centrifuged and 0.25 ml. supernatant was added to determine whether effect was due to any S I which might be dissolved from bacteria.

† Pn I S suspension was next added. Immediate flocculation took place. The large clumps formed settled rapidly. Type II serum added to the turbid supernatant caused immediate agglutination.

‡ The contents of these tubes formed large clumps, resembling those with Pn I suspension.

case of Pn II and III, presumably similar conditions of potential and cohesive force. Reagglutination is, moreover, produced almost as completely by suitable amounts of S I in solution,⁴ so that the conclusion seems inescapable that particulation, as well as the original anti-

⁴ And not by S II, the specific polysaccharide of Pn II.

gen-antibody combination, is a chemical process. The quantitative data given indicate that the particulation is a result of the original bimolecular combination as continued in a series of competing bimolecular reactions, much as in the precipitin reaction (6). It is also shown in Table VII that most of the Pn II added fails to participate in the reagglutination when Pn I is subsequently added, but may be agglutinated by pouring off the turbid supernatant and adding Type II antiserum. A similar separate agglutination of mixed microorganisms was observed by Topley, Wilson, and Duncan (17), who also concluded that the particulation phase of agglutination was strictly chemical, in confirmation of Marrack's views (14).

The results of all of these experiments, in accord with the new quantitative theory, indicate the primary importance of the chemical interaction of multivalent antigen with multivalent antibody in completing, as well as initiating, the process which the bacteriologist calls specific bacterial agglutination. If this be true, specific bacterial agglutination differs fundamentally from other instances of agglomeration and agglutination of suspended particles, and the analogy with these, so often cited, does not apply. The function of the electrolyte in specific bacterial agglutination would seem to be the secondary one of providing ions for the ionized salt complexes in which form antibody probably reacts (18), and in addition, of minimizing electrostatic effects due to the presence of many ionized groupings on the particles, effects which might interfere with the completion of particulation by chemical interaction.

Whether or not the initial bimolecular antigen-antibody reaction on the bacterial surface can take place in the absence of electrolyte, the reactants carry ionized groups and it is evident that the succeeding competing bimolecular interactions between polysaccharide molecules on partly sensitized cells and additional antibody in solution or on other cells would soon result in the formation, in the absence of electrolyte, of particles carrying large numbers of ionized groups. Coulomb forces on such particles are known to cause abnormally great viscosities and Donnan effects, so that it would not be surprising if these forces would prevent the continuation of the chemical reactions resulting in the completion of what is commonly recognized as specific bacterial agglutination. The effect of removal of salts is shown at the bottom

of Table VII. Only when the effect of these forces is eliminated by a sufficient ionic atmosphere, on addition of electrolyte, is it possible to obtain significant figures for viscosity, osmotic pressure, sedimentation constants, and the like. To ascribe a similar rôle to electrolytes in specific bacterial agglutination would seem reasonable and consistent, for after reduction of the Coulomb forces the growing particles could again interact chemically, and the process of agglutination be completed.

This conception of the effect of electrolytes is in part based on the charge-reducing properties of salts, like the older views of Bordet (19), Northrop and De Kruijff (20), Shibley (21), and others, but involves a shift of emphasis in that, regardless of any such reduction, specific agglutination takes place only when the primary chemical interaction between multivalent antigen and antibody can proceed toward completion. Many irregularities and inconsistencies in the relation of physical forces to agglutination are thus eliminated and explained.

SUMMARY

1. By the application of an absolute, quantitative microchemical method for the estimation of agglutinins, precise data have been obtained on the course of the agglutination of Type I pneumococcus by homologous anticarbohydrate.

2. Within the limitations imposed by the necessity for the agglutination reaction to take place at the bacterial surface, the reaction is shown to be analogous to the precipitin reaction and subject to the same laws.

3. The entire process of a typical instance of specific bacterial agglutination has been quantitatively accounted for on a purely chemical basis and expressed in the form of equations derived from the law of mass action.

4. Experimental verification of predictions based on the theory has shown a fundamental difference between this instance of specific bacterial agglutination and the commonly adduced analogies, and necessitated a revision of current conceptions regarding the rôle of electrolytes and of physical forces in the reaction.

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